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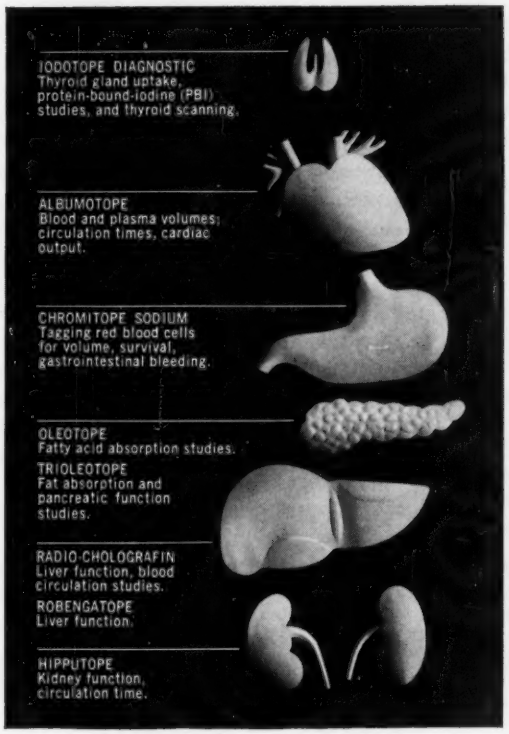

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2. In arthritis, Dianabol helps restore a sense of well-being

Plagued by pain and reduced mobility, arthritics often lose hope and become depressed. The marked improvement in general health usually associated with therapy with Dianabol may have a favorable effect in these patients, as it has in so many chronically ill individuals. As physical status improves, hope is revived and a sense of well-being restored. Commenting on the use of Dianabol in a group of debilitated, cachectic patients, Gingrich[2] states: "The majority of patients experienced increase in appetite and a feeling of well-being."

3. In arthritis, Dianabol augments the beneficial effects of salicylates, corticosteroids, etc.

Several investigators[1,3,4] have observed improved therapeutic response after the addition of Dianabol to antiarthritis regimens. Kuzell and Naugler[1] state: "In generalized osteoarthritis, symptoms have been less bothersome, and in ankylosing spondylitis gain in weight and strength has followed the use of Dianabol." Clark,[3] reporting on 12 hospitalized patients with rheumatoid arthritis being given moderate to large doses of corticosteroids with evidence of steroid intoxication, noted that the addition of Dianabol promptly decreased joint symptoms but increased steroid intoxication. However, with Dianabol it was possible to reduce corticosteroid dosage considerably, while maintaining and even furthering clinical improvement. In 15 ambulatory patients on small maintenance doses of corticosteroids, the addition of Dianabol resulted in further clinical improvement which was continued even when corticosteroid dosage was reduced in some cases.[3,4]

4. In arthritis, Dianabol counteracts the catabolic effects of corticosteroids

Prolonged use of corticosteroids may result in excessive breakdown of protein in all tissues, including bone,[5] as well as undue phosphorus and calcium loss.[6] If protein destruction is allowed to go unchecked, it may lead to osteoporosis—a condition that has occurred with increasing frequency in patients receiving corticosteroids for extended periods.[7]
Tillis[5] asserts that it is "imperative" to restore the protein bone matrix in such patients

through the use of an anabolic agent. He studied the specific anabolic benefits of Dianabol in 50 patients with osteoporosis (34 postmenopausal and 16 corticosteroid-induced), most of whom also had rheumatoid arthritis. Dianabol relieved bone pain, increased strength and vigor, and induced a sense of well-being in 41 (82 per cent) of these patients. Edema, observed in 8 patients, was cleared in 4 by reduction of dosage; the remaining 4 responded promptly to hydrochlorothiazide. Gastric distress was noted in 2 patients, slight hoarseness in 1 woman, and facial acne in 1 woman. Other investigators[8,9] have shown that addition of Dianabol to the regimens of patients receiving corticosteroids improved nitrogen and potassium metabolism and reduced phosphorus and calcium losses. Reporting on 10 patients taking corticosteroids, most of whom had corticosteroid-induced osteoporosis and/or myopathy, Abbott[9] states: "In the patients who showed a markedly negative nitrogen balance the administration of 10 mg. of Dianabol per day greatly reduced the protein deficit. In others who were eating well and taking smaller amounts of corticosteroids a positive nitrogen balance resulted which increased with Dianabol." Abbott notes that creatinuria, which occurred on corticosteroids alone, was increased by Dianabol, as it is by methyltestosterone and the newer oral methyl- or ethyl-testosterone derivatives. However, he observes that the "significance of this creatinuria is not known and no ill effects have been ascribed to this change." While the finding of elevated serum aldolase levels raised the theoretical possibility of potentially deleterious effects, Vignos *et al*[8] and Abbott[9] noted no androgenic or myopathic effects and no liver disorders in patients who took Dianabol and corticosteroids for up to 8 months. Kuzell and Naugler[1] state it is their impression that Dianabol has checked weight loss following prolonged administration of triamcinolone in patients with rheumatoid arthritis. They add that, with Dianabol, protein patterns have migrated toward normal profiles, purpura consequent to corticosteroid administration has been lessened, and the erythrocyte sedimentation rate has been diminished.

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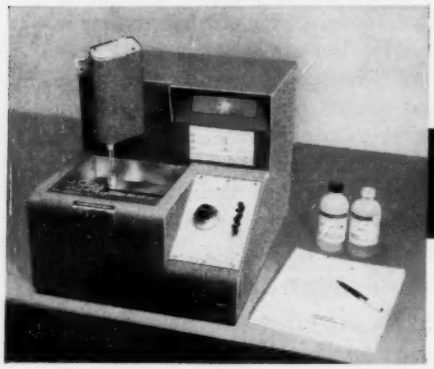

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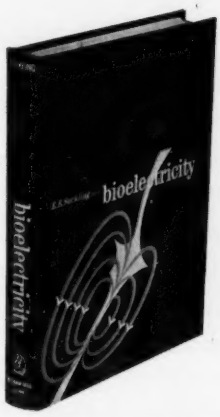

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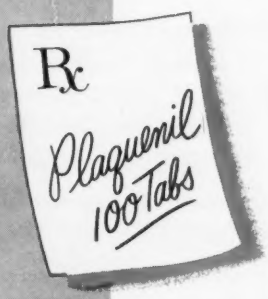

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(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

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Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

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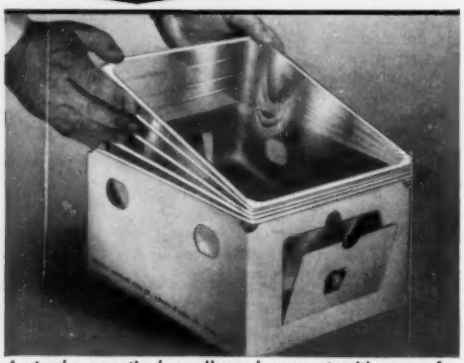

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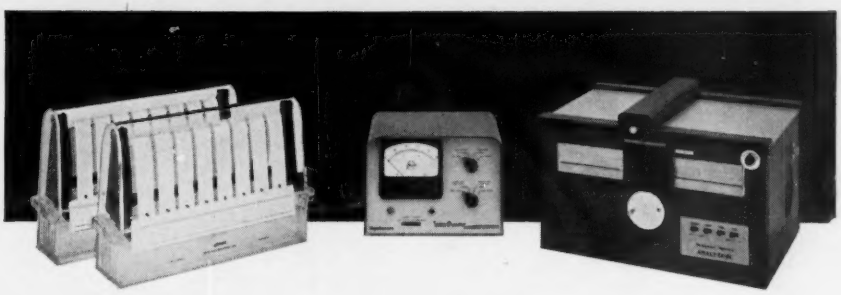

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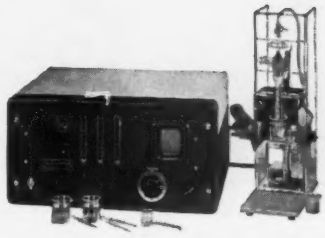

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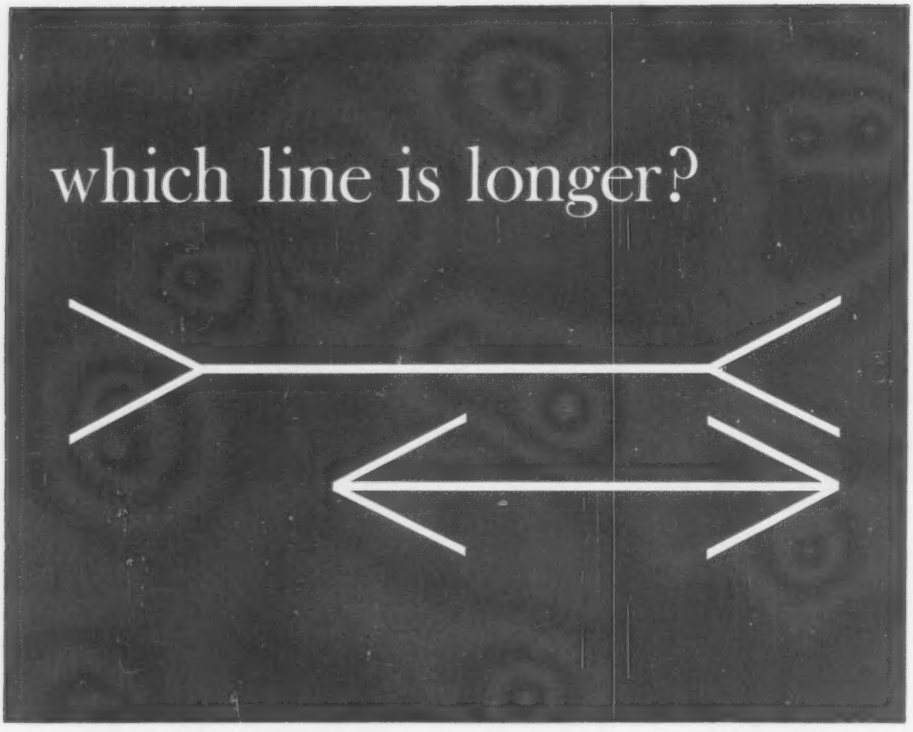

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1. Griffith, R. S.: Antibiotic Med. & Clin. Therapy, 7:129, 1960.

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IMMUNOREACTIONS INVOLVING PLATELETS. V. POST-TRANSFUSION PURPURA DUE TO A COMPLEMENT-FIXING ANTIBODY AGAINST A GENETICALLY CONTROLLED PLATELET ANTIGEN. A PROPOSED MECHANISM FOR THROMBOCYTOPENIA AND ITS RELEVANCE IN "AUTOIMMUNITY" *

By N. RAPHAEL SHULMAN, RICHARD H. ASTER, ALFRED LEITNER AND MERILYN C. HILLER

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(Submitted for publication March 8, 1961; accepted April 21, 1961)

A thrombocytopenic state developing in the absence of any apparent underlying disorder and accompanied by an abundance of megakaryocytes in the bone marrow represents the syndrome of idiopathic thrombocytopenic purpura (ITP). Similarities between this syndrome and thrombocytopenic states which can be produced experimentally with heterologous antibody (1, 2), or which occur as a result of the antigen-antibody reactions of drug purpura (1, 3, 4), suggest that an immunologic process may underlie ITP. The observations that plasma from some ITP patients, when transfused into normal individuals, may depress platelets and that plasma containing the "thrombocytopenic factor" may agglutinate platelets *in vitro* (2, 5), support the concept that an antibody may cause the disease. Because no foreign antigen has been implicated as yet in ITP, the disease is generally considered to be an "autoimmune" disorder.

The present work concerns differentiation of a specific immunologic type of purpura which heretofore was indistinguishable from ITP. The pathogenesis of the disease to be described involves mismatch of an inherited platelet antigen during blood transfusion and subsequent development of a complement-fixing antiplatelet isoantibody that is capable of producing severe thrombocytopenia in the sensitized individual.

Circumstances attending development of thrombocytopenia and characteristics of the underlying immunoreaction permitted interpretation of the mechanism responsible for destruction of autologous cells by an antibody formed against a foreign antigen, and provided a rationale for effective therapy of the disorder. This distinctive type of immune reaction is relevant to the problem of ITP and other diseases which have been considered "autoimmune" phenomena. Demonstration of the genetic control and nature of the platelet antigen responsible for post-transfusion purpura helps clarify the problems of inherited platelet types and platelet antigenicity.

CASE REPORTS

The following cases of post-transfusion purpura were studied. *Patient 1*, P.K., a white female, age 40, was admitted to the surgical service of the National Heart Institute, Clinical Center, on May 17, 1959, for correction of cardiac valvular stenosis resulting from rheumatic heart disease. Her last attack of acute rheumatic fever had been at age 17, and symptoms of cardiac failure had been progressively incapacitating for 3 years before admission. There were no other significant diseases or symptoms in her medical history; in particular, no hemorrhagic tendency and no allergic disorder. She had had three normal deliveries and pregnancies. The only drugs taken prior to admission were digitalis and chlorothiazide. Abnormalities on physical examination were limited to signs of valvular heart disease. The week prior to operation she received sulfisoxazole and tetracycline for bronchitis following transbronchial cardiac catheterization. On June 2, 1959, transventricular mitral commissurotomy was performed, during which she received two 500 ml units of whole blood and 220 ml of a third unit which was discontinued because small clots had plugged the tubing filter. The three donors were blood A_1B, Rh positive, the patient's type being A_2B, Rh positive. The operation and first 6 days of the postoperative course were uneventful, except for development of tracheitis which was treated with erythromycin and streptomycin. Early in the morning on the seventh postoperative day (June 9), the patient noticed several small hemorrhagic vesicles on her tongue and later that day developed generalized florid purpura on the trunk and extremities, including numerous 4 to 5 mm hemorrhagic bullae on the skin and mucous membranes. The same day she also had a tarry

* Part of this work was presented at the Annual Meeting of the American Society for Clinical Investigation, May, 1960, and has appeared as an abstract (J. clin. Invest. 1960, **39**, 1028).

stool, hematuria and vaginal bleeding, and developed large ecchymoses at the site of the thoracotomy incision. Other than the hemorrhagic manifestations, there were no abnormal symptoms or signs. Her platelet count on June 9 was 600 per mm^3 and her bleeding time was longer than 20 minutes; her hemoglobin was 13.4 g per 100 ml, having been 14.7 on June 4, and her white cell count and differential were normal. A unit of fresh whole blood was started the evening of June 9 but had to be stopped after 250 ml had been infused over a 1-hour period, because the patient developed severe shaking chills and slight temperature elevation. No red cell incompatibility was found, and the symptoms promptly subsided. Later that evening 250 ml of fresh platelet-rich plasma was transfused uneventfully over a 3-hour period.

Because the onset of purpura had been acute and the platelet count remarkably low, drug purpura or unusually severe ITP was the suspected diagnosis. Although the drugs being administered (digoxin, erythromycin and streptomycin) are not recognized causes of drug purpura, complement fixation and platelet agglutination tests were performed on June 10 to evaluate that possibility. The patient's serum was found to contain a factor in high titer which fixed complement with platelets from 12 normal individuals and produced marked platelet agglutination whether or not these drugs were added. Nevertheless, medications which had been given postoperatively were discontinued, and prednisone, 50 mg per day, was started. On June 10 the hemoglobin was 10.7 g per 100 ml and the platelet count was 300 per mm^3. Her bone marrow was normal with respect to the myeloid and erythroid series and contained a normal number of megakaryocytes which were almost all in the large mononuclear or megakaryoblastic form. The patient received 1,000 ml of fresh whole blood on June 10, given at a rate of 2 to 3 ml per minute, without reaction; but platelets remained at 700 per mm^3 or below. Prednisone was increased to 100 mg per day.

Because the patient had life-threatening hemorrhage, and the association of a potent antiplatelet factor with thrombocytopenia hardly seemed fortuitous, an exchange transfusion was carried out on the fourth day of purpura in an attempt to decrease the titer of the antiplatelet factor. Over a 16-hour period, a total of 5,500 ml of blood was withdrawn and 6,500 ml of fresh ACD blood replaced in 250- to 500-ml aliquots, alternating withdrawal and infusion. During the first half of the exchange, the infusion rate could not be increased above 2 to 3 ml per minute without producing marked hypotension, and during the course of the exchange the patient frequently developed urticaria which appeared to respond to intramuscular diphenhydramine. By the end of the exchange, the patient's platelet count was 4,900 per mm^3, but the bleeding time had decreased to 6 minutes. There were no further hemorrhagic manifestations, and thereafter the platelet count returned to normal with unusual rapidity, the existing purpura cleared, and the patient remained well without further treatment. The text and Figure 3 contain further details of the effects of exchange transfusion. Prednisone was tapered beginning June 13 and discontinued on June 19. On June 19 megakaryocytes in the bone marrow were normal in number and morphology. Serum protein was 6.5 g per 100 ml with 18 per cent γ-globulin on June 10 and did not change significantly after the exchange transfusion.

Patient 2, G.H., a 43 year old white female, was admitted to the Faulkner Hospital, Jamaica Plain, Mass., on September 20, 1959, with a 2-year history of peptic ulcer symptoms. There were no other illnesses, allergic disorders, or symptoms of significance in her medical history, and she had not received blood transfusions. She had had three normal deliveries and pregnancies. Because her response to medical therapy for peptic ulcer was unsatisfactory, a subtotal gastrectomy was performed on October 13, 1959, at which time she received one 500 ml unit of whole blood and 75 ml from a second unit which was stopped because of clots in the bottle. The patient's and donors' blood type was A_1, Rh positive. The postoperative course was uneventful until the sixth day, when she developed florid purpura over the entire body and hematuria. The platelet count at this time was 28,000 per mm^3, there was poor clot retraction, and the bleeding time was greater than 20 minutes. The bone marrow contained a normal number of megakaryocytes. A diagnosis of ITP was made and steroid therapy was begun. The next day, October 20, the platelet count was 6,000 per mm^3, the hematocrit was 19 per cent, and the white cell count was slightly elevated with a normal differential. Several attempts were made to transfuse fresh whole blood, but each of three units was discontinued after approximately 50 ml was received because of a reaction consisting of chills, fever, urticaria and hypotension. On both October 21 and 22 she was given the red cell content of two units of blood without reaction, the red cells having been washed to remove other cellular elements. Purpura, intermittent hematuria and hematoma formation persisted for 17 days despite high doses of prednisone. Symptoms subsided as the drug was being tapered. The clinical course is shown in Figure 1 and

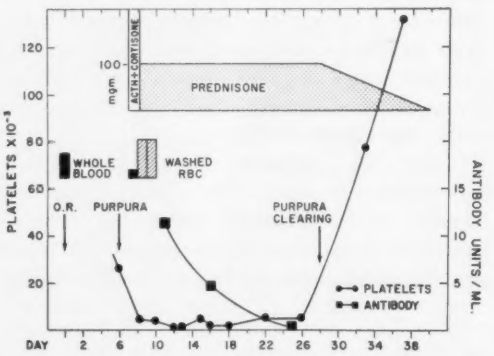

FIG. 1. CLINICAL COURSE, PATIENT 2. Units of antibody are defined in the text.

the results of tests performed with this patient's serum are presented in the text.

METHODS

Methods of *preparing platelet suspensions, counting platelets* (6) and *measuring platelet agglutination, inhibition of clot retraction,* and *complement fixation* (7) were the same as those that have been used in studying the antibody of quinidine purpura (8, 9), with the following exceptions.

The nitrogen content of platelet suspensions was determined by nesslerization. One ml of platelets at a concentration of 2×10^6 per mm^3 was found to contain 800 ± 50 μg N per ml; with this criterion for determining platelet concentration, the error was ± 5 per cent.

The 50 per cent hemolytic unit of complement activity (hereafter denoted by C′), measured by means of a single hemolysin preparation and erythrocytes from one sheep throughout this work, was equivalent to $2.5 \pm 0.2 \times 10^{-3}$ ml of reconstituted lyophilized pooled guinea pig serum. With the same reagents, the C′ content of sera from 15 normal human beings averaged 65 U per ml, with a variation of ± 10. The condition of incubation found to be optimal for maximal C′ fixation in the shortest time with the antigen-antibody system under study was 1 hour at 37° C with frequent agitation of the tubes. The rate of C′ fixation by this system was approximately halved when the incubation temperature was decreased to 27° C.

Fluorescent antibody methods. The patient's antibody-containing serum and antihuman globulin serum (Coombs serum) were conjugated with fluorescein isothiocyanate by the method of Riggs and co-workers (10) as modified by Marshall, Eveland and Smith (11) and adsorbed before use with guinea pig spleen-liver-kidney powder to decrease nonspecific fluorescence. Approximately 35 per cent of the antibody activity in the patient's serum was lost after this treatment. The fluorescein-conjugated Coombs serum used had a titer of 1/64 (see below). Other details of the techniques, such as application of antibody to platelet smears, washing procedures after exposure of smears to antibody, methods of mounting the final preparations, and the optical systems used for microscopy were the same as those reported recently by Silber and co-workers (12), with the exceptions stated in the text.

Preparation of heterologous platelet antisera. Antihuman platelet serum was made in rabbits by injecting suspensions of 3×10^9 washed platelets intravenously on Days 1, 3, 6 and 18 and was harvested on Days 11, 15, 18 and 25. The highest antibody concentration was present in all rabbits on Day 25, and serum harvested on this day was used.

Antihuman platelet serum was made in Sprague-Dawley rats by injecting suspensions of 6.3×10^8 platelets intravenously on Days 1, 3, 6, 14 and 18, and was harvested on Days 12 and 24. Serum harvested on Day 24 contained only slightly more antibody than serum harvested on Day 12.

Antihuman platelet serum was made in NIH-strain guinea pigs by injecting suspensions of 6×10^8 platelets intravenously on Days 1, 3, 6 and 9, and was harvested on Day 13. Guinea pigs were also immunized against rabbit platelets by injecting 10^9 platelets on Days 1, 3, 6, 9 and 16, and harvesting on Day 22.

Although a small amount of erythrocyte material may have contaminated platelets used for immunization, it was insufficient to produce C′-fixing anti-erythrocyte antibodies which could be detected with the maximal amount of erythrocytes contaminating platelet suspensions used experimentally. Antiplatelet serum therefore did not have to be adsorbed with erythrocytes before use. There were also no detectable antileukocyte antibodies formed that would react with the maximal amount of leukocytes contaminating platelet suspensions used in complement fixation tests.

Antihuman globulin (Coombs serum) consumption technique. A commercial preparation of rabbit antihuman serum purchased from Ortho Pharmaceutical Corp., Raritan, N. J., and preparations of horse antihuman globulin and rabbit antihuman globulin were used with identical results. In titering Coombs serum, the agglutination mixture consisted of 0.1 ml of a 1 per cent suspension of group O, Rh positive human erythrocytes sensitized with anti-D serum, plus 0.1 ml of various dilutions of Coombs serum. The titer was the limiting dilution which gave 1+ agglutination (minimal but definite macroscopic clumping) after incubation for 30 minutes at 37° C and centrifugation at 250 G for 3 minutes.

In measuring consumption of Coombs serum (13), platelets were first exposed to patients' sera containing antibody, or to normal control sera, then washed 5 times in large volumes of 0.147 M NaCl. Washing removed at most 10 per cent of antibody attached to platelets (see Section 7 in Results). After the last wash, platelets were resuspended at a concentration of 10^6 per mm^3 in saline and 0.1 ml of suspension (10^5 platelets) was mixed with 5 different progressive twofold dilutions of Coombs serum, the highest final dilution of Coombs serum being one-half the limiting dilution that gave 1+ agglutination with erythrocytes in the preliminary titration. Mixtures were incubated for 10 minutes at 37° C; platelets were then removed by centrifugation, and 0.1 ml of supernatant fluid containing residual Coombs serum was mixed with 0.1 ml of sensitized erythrocytes as described above. The amount of Coombs serum consumed by platelets was evident from the limiting dilutions which still produced erythrocyte agglutination (see Table III).

Coombs consumption tests done in an attempt to obtain evidence for a possible "blocking" antibody on platelets that were not preincubated with known antibody are referred to in the text as "direct" Coombs consumption tests. Controls for these tests were simply suspensions of platelets from normal individuals treated in the same manner as patients' platelets.

Trypsin treatment of platelets and platelet fractions. Washed platelets, prepared as described above, were suspended in 0.145 M NaCl containing 0.04 M phosphate buffer, pH 7.5, to which crystalline trypsin was added to

give a final concentration of 0.5 mg per ml. After incubation, platelets were washed three times in 0.147 M NaCl. Platelets incubated in this way for as little as 10 minutes at 37° C did not sediment as readily as untreated platelets and could not be recovered quantitatively if the usual centrifugal force was used for washing. Platelet fractions which were treated with trypsin (Section 6) were not washed; instead, the trypsin was neutralized with an equimolar amount of soybean trypsin inhibitor. The trypsin-inhibitor complex, at the concentrations used experimentally, was found to be inert in C′-fixing mixtures. Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp., and the specific activity of each preparation was assayed before use.

Measurement of sugars. Assays for total hexose by the indole and cysteine-H_2SO_4 reactions (14), for deoxy sugars by the thiobarbituric acid reaction (15), for glucuronic acid by the orcinol reaction (14), and for uronic acid by the carbazole reaction (14) were done on platelets and platelet fractions with techniques which detected with certainty 0.1 μmole of each substance assayed.

EXPERIMENTAL RESULTS

1. C′ fixation reactions of the serum factor in Patient 1 and definition of an antibody unit. Serum from Patient 1, obtained during the period of purpura, when mixed with suspensions of platelets from normal individuals, fixed C′ in the manner shown in Figure 2. No C′ was fixed when the same serum was mixed with suspensions of human erythrocytes or leukocytes.

There was no loss of activity when serum containing the factor responsible for C′ fixation was dialyzed for 72 hours at 5° C against 10^3 vol of 0.147 M sodium chloride. The factor was recovered in serum precipitates produced at one-third saturation with ammonium sulfate, solutions of which gave a visible band only in the γ-globulin region on paper electrophoresis. When serum was subjected to 76,000 G for 10 hours, by a sucrose density gradient, C′-fixing material had sedimentation characteristics of a 7S γ-globulin (16). The factor also had stability characteristics of γ-globulin in that it did not lose activity when whole or diluted serum was kept for at least 60 minutes at 56° C, 2 weeks at 5°, or for 18 months at − 20°, but did lose activity at an increasingly rapid rate when serum was incubated at temperatures above 65° or when the pH of the serum was adjusted to values above 11.2 or below 3.0 at 20°. In view of these various characteristics, the C′-fixing serum factor will hereafter be referred to as an antibody.

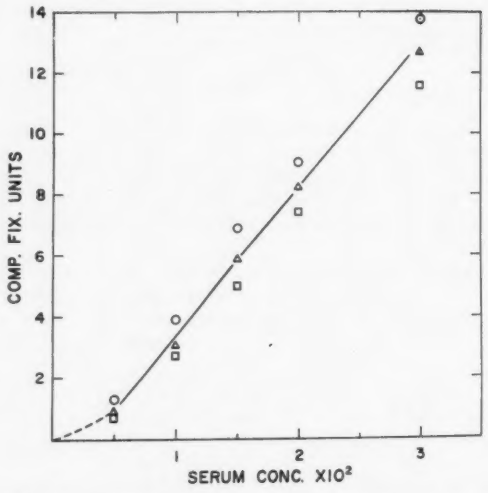

FIG. 2. COMPLEMENT FIXATION WITH VARIOUS ANTIBODY CONCENTRATIONS AND FIXED PLATELET CONCENTRATION. Fixation of C′ was carried out in a 0.3 ml volume, the mixture consisting of serum from Patient 1 obtained on June 12 before transfusion, a saline suspension of platelets to give a final concentration of 200,000/mm³, guinea pig serum (either 0.02 or 0.04 ml, containing 8 or 16 U of C′, respectively), and 0.147 M NaCl to maintain a fixed volume. Concentration of serum is expressed as ml of patient's serum per 0.3 ml reaction mixture. Each symbol represents results obtained on a different day with a platelet suspension made from a different donor, and individual points are the average of two determinations. The amount of C′ fixed when antibody and platelets were preincubated for 1 hour at 37° C, before C′ was added, was the same as the amount fixed when all three reagents were mixed together initially.

Standard curves relating the amount of C′ fixed to the concentration of antibody used (Figure 2) showed little variation in different experiments. There was no greater variation with platelets from different donors than with different platelet preparations from a single donor. In comparing antibody content of different sera, several dilutions of each sample were used, as in Figure 2, to fix amounts of C′ in the range of 2 to 12 U with platelets at 200,000 per mm³ final concentration in the reaction mixture. Although the curves obtained were not strictly linear, relative antibody content of different sera could be determined by comparing amounts of each required to fix an equivalent amount of C′, there being a simple inverse rela-

tionship between antibody content and the amount of serum necessary to fix a given amount of C′. Relative antibody concentration in different sera could be determined in this way with an error of approximately ± 10 per cent. Serum from Patient 1, obtained before the exchange transfusion, was considered the reference standard and arbitrarily assigned the value of 100 U per ml. In the linear portion of the standard curve (Figure 2), 1 U of antibody fixed 4 U of C′.

The concentration of platelets used in Figure 2 is in excess of the amount necessary to produce maximal C′ fixation by the highest concentration of antibody used. In titering antibody it was necessary to use an excess of platelets in order to obtain uniform results with platelets from random donors; for as will be seen in Figure 8, platelets from different donors in suboptimal concentrations did not give uniform results.

2. Effect of exchange transfusion in Patient 1. Figure 3 shows the effects of the exchange transfusion (see case report) on the level of circulating antibody, platelets, and serum C′. The "exchange" curve represents the dilution produced as calculated from the initial blood volume (4,900 ml) and the amount of blood removed (5,500 ml) and replaced (6,500 ml), a fixed blood volume being assumed. The increments of dilution for each 500 ml exchanged are plotted, the total exchange being approximately 70 per cent effective.

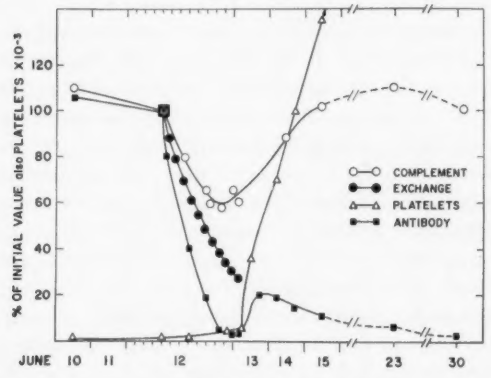

FIG. 3. EFFECTS OF EXCHANGE TRANSFUSION IN PATIENT 1. Immediately preceding the exchange, the patient's red cell volume was found to be 1,700 ml, by a Cr^{51}-labeled red cell technique. The initial hematocrit was 35 per cent and the hematocrit at the end of the exchange was 41 per cent. Antibody was assayed as described in the legend of Figure 2. Initial values for antibody and C′ were those present in the June 12 pretransfusion sample, i.e., 100 U of antibody per ml and 55 U of C′ per ml. On June 14, 250 ml of fresh whole blood was given between the two antibody determinations. On June 12 the abscissa is enlarged and divided into 2-hour intervals; the scale is interrupted between June 15 and 23, and between June 23 and 30.

Antibody level fell more rapidly than could be accounted for by simple dilution alone, for only 3 per cent of the initial concentration of antibody remained at the end of the exchange. Although replaced blood was drawn in plastic bags and stored at 5° C no longer than 12 to 24 hours, the platelet content of the transfused blood produced only a slight rise in the patient's platelet count—from 500 per mm^3 at the beginning to 4,900 per mm^3 at the end of the exchange. The C′ content of the patient's serum fell during the exchange in spite of the large amount of C′ replaced in the transfused blood. These various observations taken together suggested that antibody, combined with transfused platelets, fixed C′ *in vivo* and was sequestered with destroyed platelets.

Immediately after the exchange transfusion, the patient's platelet level rose at a rate of approximately 60,000 per mm^3 per day to reach 140,000 per mm^3 on June 15 and 350,000 per mm^3 on June 19, after which it remained at a normal level. Note that the platelet rise occurred despite reappearance of significant amounts of antibody in the patient's plasma, for although antibody level was 3 U per ml at the end of the exchange, it was 20 U per ml 13 hours later.

3. Platelet agglutination and inhibition of clot retraction by the antibody from Patient 1. In Table I the titer of the patient's C′-fixing antibody in different serum samples is compared with the titer of the platelet agglutinin and the inhibitor of clot retraction in the same samples. Since C′ fixation, agglutination, and clot retraction inhibition activities varied quantitatively in the same way in different serum samples, it appeared that the several reactions were produced by a single antibody. In support of this were the findings that amounts of normal platelets which partially or completely adsorbed the C′-fixing activity out of the patient's serum (see Figure 7) also adsorbed the agglutinin and clot retraction inhibitor to an equal degree, and that the three activities

TABLE I

*Comparison of C′ * fixation, agglutination and inhibition of clot retraction reactions †*

Sample	C′-fixing antibody	Agglutination		Inhibition of clot retraction	
		Dilution titer	C′-fixing antibody	Dilution titer	C′-fixing antibody
	U/ml		*U/ml*		*U/ml*
6–12, 1	100	1/20	5.0	1/28	3.6
6–12, 10	19	1/4	4.8	1/5	3.8
6–12, 13	5			(1/2.5)	(2.0)
6–13, 3 a.m.	3	(1/1.25)	(2.4)	(1/2)	(1.5)
6–13, 4 p.m.	20	1/3	6.7	1/5	4
6–15	10			1/2.5	4
6–23	6	1/1.25	4.8	(1/2.5)	(2.4)
6–30	2	(1/1.25)	(1.6)	(1/2.5)	(0.8)

* C′=the 50% hemolytic unit of complement activity.

† Titer of antibody by C′ fixation was measured as described in text. Titer of agglutinin expressed as the highest final dilution of patient's serum which produced 2+ agglutination (9) in reaction mixtures. Titer of inhibitor of clot retraction expressed as the highest final dilution of patient's serum which produced less than 15 per cent free fluid in mixtures with normal whole blood, dilutions being based on the plasma volume rather than the total volume of whole blood used. With this antibody, inhibition of clot retraction occurred uniformly at 37° C, but inconstantly at room temperature. The various dilutions correspond to *final* concentrations of C′-fixing antibody in units/ml as listed. Dilutions in parentheses did not produce agglutination or inhibition of clot retraction. Sample 6–12, 1 was obtained before transfusion; 6–12, 10 after 3,400 ml blood transfused; 6–12, 13 after 4,500 ml blood transfused, and 6–13, 3 a.m. at the end of the exchange after 6,500 ml blood transfused.

were recovered quantitatively in the same ammonium sulfate fraction of serum and had the same stability characteristics (see Section 1).

Platelet agglutination and inhibition of clot retraction were about equally sensitive techniques for detecting antibody. In performing platelet agglutination tests, a small volume of concentrated platelets could be added to antibody-containing serum without significantly diluting the serum, so that 0.1 ml of serum containing 0.5 U of antibody (i.e., 5 U per ml) would produce agglutination (Table I). Since a minimal final concentration of 4 U of antibody per ml (Table I) was required for inhibition of clot retraction and performance of the test necessitated diluting serum at least one-half (0.5 part antibody-containing serum to 1 part whole blood), a serum sample had to contain at least 8 U per ml to inhibit clot retraction. Although platelet agglutination was slightly more sensitive than inhibition of clot retraction, the simplicity of the latter test and the certainty of the result made it the preferable test for screening a large number of individuals for the ability of their platelets to react with the antibody (see Section 5).

C′ fixation was the most sensitive and accurate procedure for detecting and measuring this antibody, for antibody at a concentration as low as 1.0 U per ml could be measured with an error of ± 30 per cent. In measuring antibody in serum containing less than 5 U per ml, greater sensitivity was obtained by using platelets at 20,000 to 50,000 per mm^3 final concentration, rather than at 200,000 per mm^3, because relatively high concentrations of platelets produced decreases in C′ fixation (see Figure 8).

4. Reactions of the antibody from Patient 2. The initial serum sample obtained from the second patient 5 days after onset of purpura (Day 11, Figure 1) contained 11.2 U of antibody per ml, as assayed by the C′ fixation technique described above. This serum did not inhibit clot retraction and produced only equivocal platelet agglutination when used at one-third final concentration in reaction mixtures. These findings were in keeping with the relative sensitivity of C′ fixation compared with other reactions of the antibody of Patient 1. Only C′-fixation tests could be used for further evaluation of the second patient's antibody. Serum obtained 10 days after onset of purpura contained 4.2 U of antibody per ml, and antibody could not be measured in the serum obtained just before purpura cleared. It is significant that, although the first patient recovered after exchange transfusion in the face of a subsequent rise in serum antibody to 20 U per ml, the second patient had continued severe purpura when serum antibody concentration was 4.2 U per ml and less.

5. Identification of a specific inherited antigen involved in the platelet-antibody reaction. Clotted whole blood obtained from the first patient 1 day after the exchange transfusion showed partial clot retraction, the platelet count being 90,000 per mm^3; blood obtained 2 days after the exchange, when the platelet count was 140,000 per mm^3, showed normal clot retraction. This finding was unexpected, for the concentration of antibody in the patient's plasma at the time was 15 U per ml, or three times the concentration necessary to completely inhibit clot retraction of normal blood. The discrepancy proved to be due to complete refractoriness of the patient's platelets to her own antibody, a circumstance that has been reported in two other cases (17, 18).

Platelet suspensions made from Patient P.K. on June 15 (Figure 3) and thereafter did not fix C′ or agglutinate with any amount of the antibody present in her serum before or after the exchange transfusion. In addition, platelets from this pa-

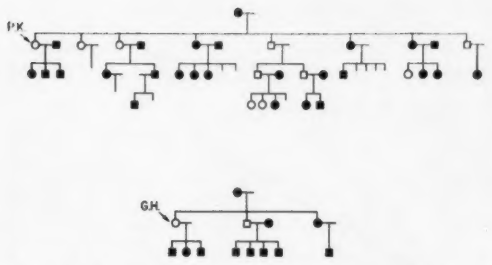

FIG. 4. INHERITANCE OF REACTIVE AND NONREACTIVE TRAITS. Circles are females, squares males. Open symbols represent individuals whose platelets did not react with the antibody in C′ fixation, agglutination and inhibition of clot retraction tests. Solid symbols represent those whose platelets reacted in all of these tests.

tient did not fix C′ with the antibody from Patient 2. Just as in the first case, platelets obtained from the second patient on Day 32 (Figure 1), shortly after recovery, did not fix complement with the antibody which was in the initial sample of her own serum and did not react with the antibody from Patient 1 in C′ fixation, platelet agglutination, and inhibition of clot retraction tests. When platelets were obtained from the patients' relatives, it was apparent that failure of the patients' platelets to react was not owing to an interfering substance in the patients' recovery sera or on their platelets,[1] but to lack of an inherited antigen against which the antibody had developed.

Figure 4 demonstrates the manner in which reactive platelets (solid symbols) and nonreactive platelets (open symbols) are inherited. The antigen appears to be a dominant mendelian character; but as will be seen, the reactive trait does not fit the strict definition of dominance which demands that the character be as fully expressed when the gene is in the heterozygous state as it is when the gene is in the homozygous state. The reactive trait—i.e., the gene responsible for the antigen that reacted with the antibody—will be labeled Pl^{A1}, and the nonreactive trait, Pl; reasons for this notation will be discussed.

Of the 206 randomly selected normal individuals subsequently tested, 3, or 1.5 per cent, had nonreactive platelets. Moreover, it was found that reactive platelets could be separated into two groups based on differences in the amount of C′ fixed per platelet (Figure 5).

The over-all variation in C′ fixation observed when suboptimal concentrations of platelets from different normal donors were used was much more than could be accounted for on the basis of errors in measuring C′ fixation (cf. Figure 2) or in counting platelets. Platelet activities fell into two groups without overlap, even though there was considerable spread of values in each group. The

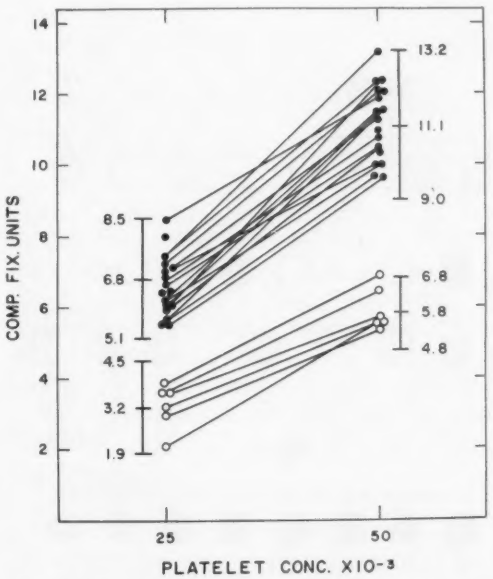

FIG. 5. QUANTITATIVE ACTIVITY OF PLATELETS FROM RANDOM DONORS. Platelets were prepared in the usual way and counted with 3 pipets and 6 chambers. The two final platelet concentrations used were 25,000/mm³ and 50,000/mm³ in reaction mixtures containing 4 U of antibody. This amount of antibody fixed 15 to 17 U of C′ in the presence of an excess (200,000/mm³) of any donor's platelets (see Figure 2). The brackets delimit 2 standard deviations from the mean for each group. The graph is a composite of results obtained with different donors on different days.

[1] Because platelets from all normal individuals tested up to this point had reacted with the antibody from both patients, numerous attempts were made to detect a possible "blocking" antibody on the patients' platelets. The general techniques used were the direct Coombs test, "direct" Coombs consumption, and various elution procedures, as well as incubation of antibody or normal platelets with patients' recovery sera before completing usual C′ fixation or agglutination reaction mixtures. None of the techniques gave presumptive evidence for a blocking antibody on platelets or a factor in serum which would inhibit the activity of the original antibody; but with all of these techniques, it is impossible to draw definite conclusions from negative results.

intragroup spread could be decreased by preparing suspensions of platelets from different donors simultaneously, by performing C′ fixation tests with all platelets on the same day, and by using nitrogen content of platelet suspensions rather than counts as the criterion of platelet concentration. With these refinements, the values for standard deviations shown in Figure 5 were approximately halved, but the mean values for the two groups remained essentially the same. Thus, of 23 randomly selected individuals tested, 74 per cent had platelets which fixed 1.7 to 2.4 times more complement than did platelets from the remaining 26 per cent.

If inheritance of the antigen were controlled by a pair of alleles, all children of a parent who had nonreactive (Pl Pl) platelets would have at least one *Pl* gene; and if the children's platelets were reactive, their genotype would have to be Pl^{A1} *Pl*. Quantitative C′-fixing activity of platelets from five family groups containing a nonreactive parent and reactive children is shown in Figure 6. In all 11 instances (children in Families 1 to 5) there was 100 per cent correlation between low-activity platelets and the postulated heterozygous state. Three mates of individuals with nonreactive platelets had high-activity, presumably Pl^{A1} Pl^{A1}, platelets; all seven children (in Families 1, 3, and 4) were apparent heterozygotes. In Family 2, the mate of a nonreactor had low-activity, presumably Pl^{A1} Pl, platelets, and two of the three children tested were nonreactors, the third being an apparent heterozygote. Because, in all families tested, there was strict correlation between platelet activity and the postulated mode of inheritance, random donors whose platelets gave low activity were most likely Pl^{A1} *Pl* individuals, and those whose platelets gave a high activity were most likely Pl^{A1} Pl^{A1}. In Family 6, the mother and eight children had high-activity platelets; this would be the circumstance if both parents were genotype Pl^{A1} Pl^{A1}.

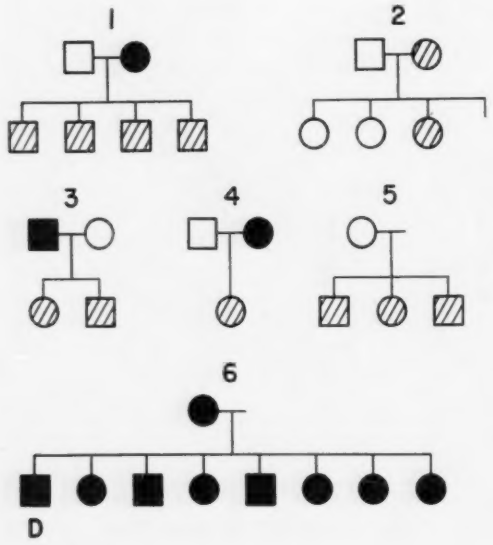

FIG. 6. QUANTITATIVE ACTIVITY OF PLATELETS FROM FAMILY GROUPS. Individuals whose platelets, at a final concentration of 50,000/mm³, fixed less than 5.8 U of C′ in the presence of excess antibody are represented by hatched symbols and those whose platelets fixed greater than 9.2 U of C′ under the same conditions are represented by solid symbols (see Figure 5). No values for C′ fixation were obtained in the range of 5.8 to 9.2 U. Open symbols represent individuals whose platelets did not react. Family 6 was the family of the individual (D) who donated blood to Patient 2.

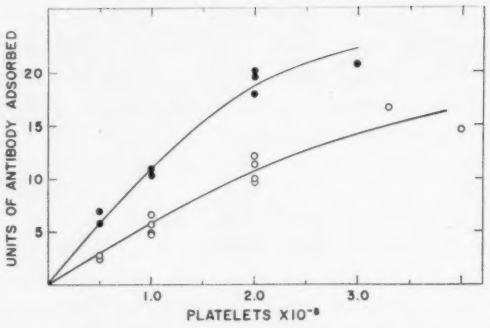

FIG. 7. ADSORPTION OF ANTIBODY BY HIGH-ACTIVITY AND LOW-ACTIVITY PLATELETS. Buttons prepared from suspensions of high-activity and low-activity platelets were resuspended in 0.25 ml serum containing 25 U of antibody. The mixtures were incubated for 90 minutes at 37° C, centrifuged at 15,000 G for 15 minutes, and antibody remaining in the supernatant serum was titered as described in Figure 2, using the average results of 3 different dilutions. Individual points for each amount of platelets used were obtained with platelets from a different donor. The C′-fixing activity of the platelets (● high-activity, ○ low-activity) was determined as described in Figure 5.

The amounts of antibody adsorbed by the different types of platelets are compared in Figure 7. Nonreactive platelets at any concentration did not adsorb antibody. High-activity, presumably Pl^{A1}

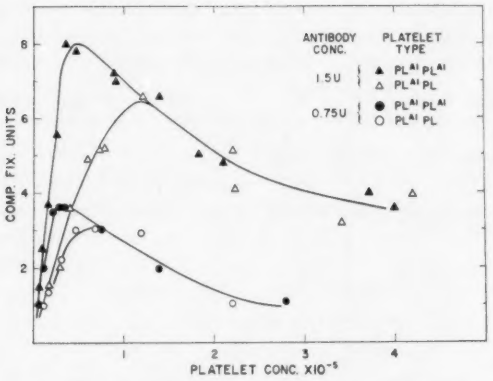

FIG. 8. EFFECTS OF VARYING THE CONCENTRATION OF THE TWO TYPES OF PLATELETS WITH ANTIBODY CONCENTRATION FIXED. Amount of C′ fixed was determined as in Figure 2. Reaction mixtures contained either 0.015 or 0.0075 ml of patient's serum in a final volume of 0.3 ml. The various final concentrations of Pl^{A1} Pl^{A1} or Pl^{A1} Pl platelets used with each amount of serum are indicated on the abscissa.

Pl^{A1}, platelets adsorbed approximately twice as much antibody per platelet as did low-activity, presumably Pl^{A1} Pl, platelets. In partially adsorbed serum samples, decreases in titer of agglutinin and inhibitor of clot retraction corresponded to decreases in titer of C′-fixing antibody.

Because of the variation in platelet activity, it was not possible to establish an alternate antibody unit by determining the minimal concentration of platelets required for maximal C′ fixation, as has been done in the case of the quinidine purpura antibody (8). Titrations of this type (Figure 8) with platelets of different activity gave approximately a twofold difference in the platelet concentrations necessary to produce maximal C′ fixation with a single concentration of antibody. However, in the presence of an excess of either type of platelet, a single concentration of antibody fixed the same amount of C′. Either type of reactive platelet could be used with uniform results when antibody was assayed as in Figure 2.

From the data shown in Figures 5, 6, 7 and 8, it was evident that three platelet phenotypes could be differentiated which corresponded most likely to the genotypes *Pl^{A1} Pl^{A1}*, *Pl^{A1} Pl* and *Pl Pl*.

Differences evident in the C′-fixation reaction were not detectable by agglutination or inhibition of clot retraction techniques, for within the limits of error, both types of reactive platelets were agglutinated and their clot retraction activity inhibited to an equal degree by the same final effective concentrations of antibody. There were no microscopic morphologic differences between platelets that could be identified as phenotypes Pl^{A1} Pl^{A1}, Pl^{A1} Pl, or Pl Pl, no differences in the mean volume determined by a microhematocrit method, and no differences in the nitrogen or total hexose content (see Section 6) per platelet. Sera of all normal individuals with Pl Pl platelets showed no evidence of a naturally occurring antibody against Pl^{A1} antigen by agglutination and C′ fixation tests; similarly, sera of individuals with Pl^{A1} Pl^{A1} platelets had no detectable antibody against Pl Pl platelets. There was no correlation between the different platelet types and the presence or absence of erythrocyte antigens, A_1, A_2, A_3, B, O, M, N, S, P, C, D, E, c, e, D^u, K and Fy^a, based on typing erythrocytes from a total of 52 individuals (22 with Pl^{A1} Pl and 12 with Pl Pl platelets). All individuals tested had erythrocyte antigens s and k and lacked antigens C^w and Di^a. The secretor trait was found to be either present or absent in individuals with Pl^{A1} Pl^{A1}, Pl^{A1} Pl, or Pl Pl platelets.

6. The nature of the Pl^{A1} antigen. The Pl^{A1} antigen on platelets suspended in 0.147 M NaCl retained full ability to fix C′ with antibody after at least a month in the frozen state at $-20°$ C, after repeated freezing and thawing, and after incubation for at least 5 hours at 37° C, but lost 3 to 4 per cent of remaining activity per day at 5° C. Neither exposure to hypotonic and hypertonic solutions (i.e., distilled water to 2.56 M NaCl) for at least several hours at 20° C, nor prolonged incubation at pH values from 4.8 to 9.4 affected the C′-fixing activity of the antigen.

Although platelets in ACD whole blood stored under blood bank conditions for 20 days were not morphologically intact, approximately 50 per cent of the initial Pl^{A1} antigen could be recovered in suspensions of particulate material made in the same way as platelet suspensions from fresh blood. In an occasional blood sample, some of the particulate antigen-containing material which was present in plasma after storage for 10 to 30 days at 5° C would not sediment at 80,000 G, but this material could be removed by filtering plasma through a 300 mμ pore-size Millipore filter.

Suspensions of reactive platelets heated at 56° C

TABLE II

Preparation and properties of platelet fractions *

Platelet preparations	Characteristics	C′-fixing activity		N content		Sugar content	
		U/ml	%	*μg/ml*	%	*μg/ml*	%
No. 1: Suspension of Pl^{A1} Pl^{A1} platelets in saline, 1.5×10^6/mm³	Turbid, not transparent	903	100	577	100	219	100
No. 2: Prep. 1 treated in Raytheon 9 KC oscillator at max. voltage and frequency for 7 min at 3° C	Transparent opalescence, no visible platelets at 800 ×	866	96	577	100	219	100
No. 3: Prep. 2 centrifuged 8,000 G for 15 min; supernatant fluid assayed	Crystal clear	650	72	462	80		
No. 3A: Alternately, Prep. 2 filtered through 1 μ sintered glass filter; filtrate assayed	Crystal clear	497	55	363	63		
No. 4: Prep. 3 (i.e., clear superantant fluid) centrifuged 80,000 G for 45 min; precipitate resuspended in original volume 0.147 M NaCl and assayed	Yellow button; suspension in original vol clear; in 1/10 original vol, opalescent	268	30	47	8	21	9
Supernatant fluid of no. 4	Clear	9	1	335	58		

* C′-fixing activity of each preparation determined as shown in Figure 8 by using several dilutions of antigen with excess antibody. Nitrogen determined by nesslerization. Sugar expressed as μg glucose based on total hexose assay by indole reaction after hydrolysis with conc. H_2SO_4 for 20 minutes at 100° C. Particulate material obtained in Prep. 2 when oscillation time was less than 3 minutes did not pass a 1 μ pore-size filter, and when greater than 9 minutes, the C′-fixing activity of the sonicate decreased progressively. Concentration of platelets had to be less than 2×10^6/mm³ for most effective disintegration to occur in the oscillator.

for 10 minutes, incubated at pH 3.2 or 10.0 for 30 minutes at 20° C, and incubated in 50 per cent ethanol-water for 10 minutes at 20° C lost completely and irreversibly their ability to fix C′ in the presence of antibody. However, these forms of treatment had relatively little effect on the ability of platelets to combine with antibody, for the same platelets which had lost their C′-fixing property were at least 80 per cent as effective as untreated platelets in adsorbing antibody (see Figure 7). True inactivation of the antigen (i.e., loss of both C′-fixing and antibody-binding properties) was not complete after heating at 56° C for 2 hours, but was almost complete after heating at 100° C for 10 minutes or after incubation in 50 per cent acetone-water for approximately 15 minutes.

The antigen on platelets or in a particulate fraction of platelets (preparation 4, Table II) retained full activity when incubated in trypsin at a concentration of 0.5 mg per ml for up to 4 hours at 37°, in addition to 16 hours at 25° C. The antigen appeared to be associated with the stromal fractions of platelets. To prepare platelet stroma, platelet suspensions were frozen and thawed three times, and material that sedimented in 15 minutes at 14,000 G was then incubated for 1 hour in distilled water and washed three times in 0.147 M NaCl (8). Several stroma preparations of this type contained approximately 30 per cent of the nitrogen and 70 per cent of the complement-fixing activity present in the initial suspension.

Some further purification was obtained by exposing suspensions of whole platelets or platelet stroma to ultrasonic vibration and separating antigen from residual platelet material by differential high speed centrifugation or fine filtration. Table II lists the conditions of preparation and properties of the sonicate.

The ratio of C′-fixing activity to N content, as well as to sugar content, could be increased four- to fivefold by these procedures. Suspensions of preparation 4, which contained 12 μmoles of hexose (as glucose) per ml, had no detectable uronic acid, glucuronide, or deoxy sugars. C′-fixing material of preparation 4 was in particulate form which passed a 300 mμ pore-size filter but was 95 per cent retained by 50 mμ pore-size. Pl^{A1} Pl platelets treated in the same way as Pl^{A1} Pl^{A1} platelets gave approximately 50 per cent the yield of C′-fixing material at each step of fractionation, and Pl Pl platelets gave none.

Antigen in platelet fractions had stability properties similar in all respects to those described above for the antigen on platelets. Moreover, the

relationship between the amount of C′ fixed and the amount of antibody bound by platelet fractions was quantitatively the same as in the case of whole platelets—i.e., curves in Figures 7 and 8 could be reproduced with the fractions. Preparation 4, Table II, did not produce a precipitin reaction when mixed in suboptimal, optimal or above optimal amounts with serum containing 100 U of antibody per ml, or when layered on serum containing this concentration of antibody. Nonreactive Pl Pl platelets, incubated in preparation 4 and washed once, did not acquire the ability to fix C′.

7. The nature of the antigen-antibody complex. The following experiments indicated that the antigen-antibody complex was very stable. Platelets in complexes formed in the presence of excess antibody could be washed four times in distilled water or various concentrations of NaCl up to 2.56 M at temperatures from 0° to 40° C and remain at least 90 per cent saturated with antibody. There was no detectable antibody in supernatant fluid of complexes heated for as long as 120 minutes at 56° C in 0.147 M or 2.5 M NaCl, although any free antibody which might have been released would have been stable under these conditions (Section 1). The supernatant fluid of complexes incubated for 30 minutes at 20° C in 0.147 M NaCl adjusted to pH 10.1 with NaOH, then neutralized with HCl after sedimenting platelets did not contain detectable free antibody, although antibody was stable at this pH (Section 1). Thus, heat and high pH, which completely abolished the ability of complexes to fix C′, did not effect release of antibody.

Of the various techniques tried, only incubation at low pH produced significant dissociation of platelet-antibody complexes. The optimal pH for maximal dissociation was found to be 3.1 in 0.147 M NaCl adjusted with HCl; the maximal yield of free antibody was 18 to 23 per cent of the amount attached to platelets. Platelet material was separated from the acid medium by centrifugation, and the supernatant fluid was neutralized before being assayed. Most of the antibody that would elute at pH 3.1 did so within 15 minutes at 20° C. Prolonged incubation at pH 3.1 at other temperatures from 0° to 56° C and in 2.56 M NaCl did not increase the yield of free antibody. At pH values below 3.0, antibody inactivation (see Section 1) apparently proceeded along with elution, for the yield of free antibody was progressively less; at pH values above 3.4, no free antibody was obtained.

8. Antiglobulin consumption and fluorescent antibody studies. Typical results of experiments with Coombs serum are shown in Table III. Nonreactive platelets incubated in normal serum (column 3) or in patient's serum (column 4) and reactive platelets incubated in normal serum (column 5) contained sufficient nonspecifically adsorbed material after five washes so that 10^8 platelets consumed approximately that amount of antihuman globulin contained in 0.1 ml Coombs serum with a titer of 1/2 (see Methods). Pl^{A1} Pl^{A1} platelets saturated with antibody consumed more Coombs serum (column 6) than did control platelets. Approximately 10 U of antibody attached to 10^8 platelets (see Figure 7) appeared to account for the specific binding of that amount of antihuman globulin contained in 0.1 ml of Coombs serum with a titer of 1/4. Approximately the same amount of Coombs serum was adsorbed by 10^8 erythrocytes saturated with anti-D.

Fluorescent antibody methods which have been used to study reactions between platelets and heterologous antiplatelet antibodies (12, 19) were applied to the present antigen-antibody system with both the direct and indirect "staining" techniques. In the direct method, fluorescein-conjugated patient's serum was applied to platelet

TABLE III

Fixation of antihuman globulin by platelet-antibody complexes *

1	2	3	4	5	6
Coombs serum, final dilution	Preliminary titration	Pl Pl platelets + normal serum	Pl Pl platelets + patient's serum	Pl^{A1} Pl^{A1} platelets + normal serum	Pl^{A1} Pl^{A1} platelets + patient's serum
1/40	4+	4+	4+	4+	2+
1/80	4+	3+	4+	3+	2+
1/160	3+	3+	3+	2+	1+
1/320	3+	3+	2+	2+	0
1/640	2+	1+	1+	1+	0
1/1,280	1+	0	0	0	0

* General technique described in Methods. The initial adsorption mixtures consisted of 10^8 platelets suspended either in 1 ml of patient's serum containing 100 U of antibody/ml, or in 1 ml of normal serum from an individual with type AB erythrocytes. After incubation for 30 minutes at 37° C and 5 washes, 10^8 platelets from each mixture were suspended in 0.2 ml of Coombs serum at 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 dilutions. Supernatant Coombs serum from these mixtures was then added to an equal volume of a suspension of sensitized erythrocytes to give the final dilutions of Coombs serum listed in column 1. The preliminary titration of plain Coombs serum is shown in column 2. In columns 3, 4, 5 and 6 the initial adsorption mixtures used and the degree of agglutination obtained are listed. Similar results were obtained in 4 separate experiments with platelets from a different donor each time.

smears and incubated for 60 minutes at 37° C; excess serum was then washed off and the slide dried and mounted. In all instances the content of antibody in the conjugated serum used was at least 20-fold that necessary to saturate platelet smears; smears were used immediately after being dried in air, not fixed by organic solvents. The washing procedures necessary to remove excess serum were known not to dissociate antigen-antibody complexes (see Section 7), but the degree of fluorescence shown by preparations made with Pl^{A1} Pl^{A1} platelets could not be distinguished from background or from that shown by similar preparations made with Pl Pl platelets. In the indirect technique, unconjugated serum containing antibody in great excess of that necessary to saturate the antigen was applied to platelet smears or mixed with platelet buttons. Platelet smears coated with anti-Pl^{A1} serum or platelets suspended in the serum were incubated for 60 minutes at 37° C. Smears were washed after incubation and coated with fluorescein-labeled Coombs serum. Suspensions were washed after incubation, then smeared on slides and coated with Coombs serum. In each case Coombs serum was kept on the slides for 60 minutes at 20° C, then washed off. Again, none of the preparations with Pl^{A1} Pl^{A1} platelets showed fluorescence that differed from that of control preparations.

9. Reactions of patient's antibody with heterologous platelets. Quantitative C′-fixation activity of platelets obtained from various animals was determined, the same final concentrations of platelets and the same concentration of antibody as in Figure 5 being used. Platelets from 11 mongrel dogs, when used at a concentration of 25,000 per mm^3, fixed 4 to 7 U of C′, and at 50,000 per mm^3, fixed 7.5 to 11 U of C′. The dogs were therefore a homogeneous group with respect to platelet activity, and the activity was comparable with that of Pl^{A1} Pl^{A1} human platelets. Platelets from 25 rabbits, all from different litters, fixed C′ with the patient's antibody. Rabbits also appeared to be a homogeneous group with respect to platelet activity; for at a concentration of 25,000 per mm^3, rabbit platelets fixed 2 to 4 U of C′, and at 50,000 per mm^3, fixed 5 to 8 U. Relatively low activity (cf. Figure 5) of the apparently homozygous rabbit platelets was probably due to their very small size, for the hematocrit of rabbit platelets in a suspension containing 2×10^6 per mm^3 was 6 vol per cent, whereas the hematocrit of the same concentration of human platelets was 15 vol per cent. Platelets from 3 Cynamacus and 3 Rhesus monkeys reacted with the antibody. Quantitative activity of monkey platelets was relatively low on the basis of cell count, but again, monkey platelets are smaller than human platelets. Animal platelets which fixed C′ with the patient's serum adsorbed all of the antibody that reacted with human platelets and the reverse was also true. None of the animal platelets tested reacted with normal human serum to produce C′ fixation.

Although complexes of dog platelets with patient's antibody fixed guinea pig C′ in amounts similar to that fixed by human platelet-antibody complexes, the patient's antibody, in concentrations up to 20 U per ml, would not inhibit clot retraction of dog blood (cf. Table I). The discrepancy between the ability of dog platelets to react with antibody and the inability of antibody to inhibit clot retraction of dog blood may be explained by failure of dog C′ to be fixed as effectively as human C′ by platelet-antibody complexes; for platelet-antibody complexes fixed very little, if any, dog C′ when dog serum was substituted for guinea pig serum as a source of equivalent C′ activity in reaction mixtures. The same discrepancy was observed in the case of rabbit blood.

Although all dog, rabbit and monkey platelets tested reacted with the antibody, platelets from 16 Sprague-Dawley rats and 12 NIH-strain guinea pigs, and thrombocytes from 6 New Hampshire chickens did not react with the patient's antibody in C′ fixation tests and did not adsorb antibody from the patient's serum.

10. Reactions of heterologous antisera with human platelets. Antisera against human Pl^{A1} Pl^{A1}, Pl^{A1} Pl, and Pl Pl platelets were made both in animals whose platelets contained the Pl^{A1} antigen (10 rabbits) and in animals whose platelets lacked the Pl^{A1} antigen (11 rats and 9 guinea pigs). Regardless of the animal used or the type of human platelet against which it was sensitized, the antibody that formed reacted with the three types of human platelets to give the same quantitative relationships between the amount of C′ fixed per platelet in the presence of a fixed concentration of antibody (e.g., Figure 9, curve 1), and between the amount of C′ fixed by increments of antibody

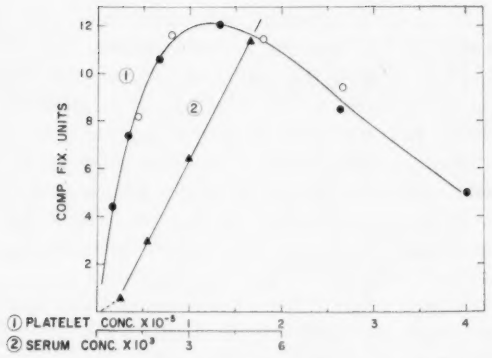

FIG. 9. COMPLEMENT FIXATION WITH HETEROLOGOUS ANTIBODY. Curve 1 is comparable with curves obtained in Figure 8. Reaction mixtures contained a fixed amount (0.005 ml) of serum from a rabbit sensitized with Pl^{A1} Pl^{A1} platelets. The solid circles are results obtained with the same platelets used for sensitization, and the open circles obtained with Pl Pl platelets. Final concentration of platelets in the mixtures are on the abscissa. Curve 2 is comparable with the curve in Figure 2. The reaction mixture consisted of a fixed final concentration (100,000/mm^3) of the same platelets that had been used for immunization and various concentrations of the same immune rabbit serum used to obtain curve 1. Concentration of serum is expressed as ml of rabbit serum per 0.3 ml reaction mixture. Similar results were obtained with all antihuman platelet sera, but antibody titers in different animals varied from 10 to 625 U/ml, a unit being defined in the same way as a unit of patient's antibody.

in the presence of a fixed concentration of platelets (e.g., Figure 9, curve 2). Moreover, antibody made in rabbits, rats, or guinea pigs against any one of the three types of human platelets was adsorbed completely by each type of human platelet, leaving no residual antibody that would react with the other two types. Thus, neither the Pl^{A1} antigen on human platelets injected into animals which lacked the antigen on their platelets, nor the possible antigen "allelic" to Pl^{A1} (see Discussion), injected into animals which were apparently homozygous for the Pl^{A1} antigen, provoked a type-specific antibody. Since these experiments involved a total of 28 antisera made against and cross-adsorbed with platelets from 18 different human beings, it appears either that those human platelet antigens which provoke C′-fixing antibodies in animals are shared by many individuals, or that the animals used do not discriminate (20) between type-specific antigen and other human platelet antigens.

11. In vivo effects of the patient's antibody in animals and human beings. In view of the finding in Patient 2 (Figure 1) that thrombocytopenia persisted when antibody concentration was too low to be detected by *in vitro* techniques, it appeared that very little of the antibody from Patient 1 would be necessary to produce thrombocytopenia experimentally *in vivo*. The plasma used for all *in vivo* tests was obtained from Patient 1 and contained 72 U of antibody per ml. One ml of plasma injected intravenously in an individual who had Pl^{A1} Pl^{A1} platelets did not change the platelet count. As shown in Figure 10, 5 ml of plasma containing 360 U of antibody, given to the same individual, depressed the platelet count 60,000 per mm^3 below the baseline value within 90 minutes, and a sustained depression of 40 to 50,000 per mm^3 lasted for approximately 24 hours. A second individual who had Pl^{A1} Pl^{A1} platelets was given 10 ml of plasma intravenously; platelets gradually fell from 170,000 per mm^3 to 50,000 within 2 hours and remained below the baseline value for more than 2 days. There were no ill effects whatsoever

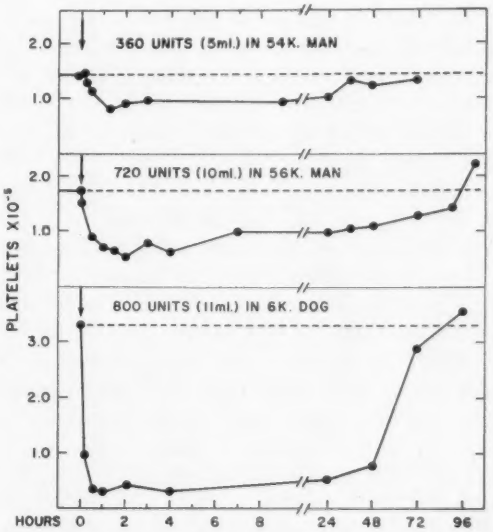

FIG. 10. *In vivo* EFFECTS OF PATIENT'S ANTIBODY. All platelet counts were performed with a minimum of 4 pipets and 8 chambers, with venous blood. The same plasma sample was used in all experiments and was titered for antibody just before use. The dog was not anesthetized and plasma given to the dog had been heated at 56° C for 30 minutes. Horizontal dashed line is the baseline platelet value.

after administration of the antibody and no change in the leukocyte count or serum C′ content.

Antibody which produced the decrease of 120,000 platelets per mm^3 *in vivo* could not be detected in the recipient's blood by *in vitro* measurements. The 720 U would have resulted in a final plasma concentration of approximately 0.35 U per ml on the basis of dilution alone, and several minutes after injection free antibody could not be detected in the recipient's serum by C′ fixation techniques. Moreover, any antibody adsorbed on circulating platelets was too small an amount to detect by agglutination, inhibition of clot retraction or Coombs consumption tests, with the recipient's own platelets or whole blood being used.

One year after Patient P.K. had recovered, she was given 68 ml of her own plasma containing a total of 4,900 U of antibody assayed before injection. Although the amount of intravenous antibody given was 13 times greater than had been used to depress platelets in an individual with PI^{A1} PI^{A1} platelets, her platelet count remained unchanged. The antibody did not produce ill effects or change her white cell count.

The antibody was not so effective in dogs as in man. As much as 3.5 ml of plasma (250 U of antibody) given to a dog weighing 6 kg did not produce a sustained thrombocytopenia, but 11 ml of plasma (800 U of antibody) produced marked and lasting thrombocytopenia (Figure 10). A second dog given the same dose, 133 U (1.8 ml) of antibody per kg, had an identical response. The dogs did not have hemorrhagic symptoms, discernible ill effects, change in leukocyte count, or a decrease in serum C′. Because several normal human sera, as well as the patient's serum, contained an agglutinin for dog platelets which was present in dilutions up to 1/10 after adsorption with human platelets, the same dogs were also injected with the patient's plasma from which anti-PI^{A1} had been adsorbed completely with human PI^{A1} PI^{A1} platelets, as well as with plasma obtained from the patient 6 months after recovery. These control injections (2 ml of plasma per kg) produced approximately a 30,000 per mm^3 decrease in platelet count which lasted less than 1 hour. Control injections were given in one dog 3 days before antibody, and in the other dog, 4 days after antibody.

12. Other observations in human beings on mismatch of PI^{A1} and the possible antigen "allelic" to PI^{A1}. During the period of purpura, the first patient received a total of 17 U of fresh whole blood and the second patient received small amounts of fresh blood from three different donors, without evidence of further provocation of antibody in each case. It was considered that neutralization of transfused antigen by remaining antibody may have prevented stimulation of antibody production in these instances, and that antigen might be effective if given after antibody had disappeared. However, after administration of various forms and amounts of antigen (listed in Table IV) to Patient 1 long after disappearance of detectable antibody, all tests for C′ fixing and agglutinating antibodies remained negative, as did tests for possible "blocking" antibody with the direct and indirect Coombs consumption techniques. There was also no evidence of a "block-

TABLE IV

Attempt to provoke anti-PI^{A1} antibody in a previously sensitized individual *

Infusion no.	Interval after recovery	Material infused
1	7 mos.	PI^{A1} PI^{A1} platelets from 500 ml fresh ACD blood, washed once in saline
2	8 mos.	500 ml PI^{A1} PI^{A1} whole blood stored 1 week
3	10 mos.	25 ml PI^{A1} PI^{A1} platelet-rich plasma, stored 5 days, from the donor to Pat. 2
4	10 mos. + 11 days	200 ml fresh whole blood from a donor to Pat. 1
5	10 mos. + 17 days	125 ml platelet-rich plasma, stored 22 days, from the donor to Pat. 2
6	14 mos.	Fractionated PI^{A1} PI^{A1} platelets, Prep. 4 in Table II, 32% yield from total of 5×10^{10} platelets
7	18 mos.	225 ml whole blood, stored 3 days, from the donor to Pat. 2

* The recipient in each instance was Patient 1. The donor to Patient 2 was the individual who supplied the one full unit of blood for the transfusion at operation, and the donor to Patient 1 was one of three whose blood the patient had received during operation (see Case Reports). None of the infusions produced symptoms and the recipient's platelet count did not change during postinfusion periods. Tests for antibody, by a C′-fixation technique that could detect 1.0 U of antibody/ml performed on sera obtained daily for 8 to 12 days after each infusion, were negative. The same sera were also used in agglutination tests and in various tests for possible "blocking" antibody (see text). After infusions 1 and 7, a "direct" Coombs consumption test was done, using the recipient's own platelets. All of these tests were negative.

TABLE V

Comparison of patients who developed purpura after transfusion

Sex	Age	Initial transfusion	Post-transfusion thrombocytopenic state				
			Onset	Duration	Megakaryocytes	Transfusion reaction	Treatment
			days	*days*			
F	40	3 U*	7	3	plentiful	+	Exchange transfus.
F	42	1 U† (75 ml of 2nd)	6	20	plentiful	+	Steroids
F	56(17)	1 U‡	5	17	plentiful	+	Steroids
F	51(18)	1 U	7	8	increased	+	None

* Each unit stored 20 days in blood bank; small clots in one unit.
† Each unit stored 16 days in blood bank; large clots in second unit.
‡ Blood stored 10 days in blood bank (personal communication from M. Zucker).

ing" antibody which might have been detected by its inhibitory effects in C′-fixation reactions; for 10^8 Pl^{A1} Pl^{A1} platelets, incubated in 1.0 ml of the various post-transfusion sera (see legend, Table V) for 1 hour at 37° C, retained full ability to react with C′-fixing antibody, and the activity of small amounts of C′-fixing antibody (0.5 to 1.0 U) was not diminished in the presence of 0.25 ml of the various post-transfusion sera. Moreover, there was no indication that hypersensitivity of the delayed type had developed, for the infusions of antigen caused no systemic symptoms and intradermal injection of antigen produced no local reaction.

Of the large group of normal individuals whose platelets were tested with anti-Pl^{A1}, two whose platelets were nonreactive (Pl Pl) had received several transfusions during operative procedures 2 and 3 years before, respectively. They had not developed hemorrhagic complications after transfusion, although the chances were at least 50 to 1 that each unit of blood they received contained Pl^{A1} antigen.

A normal individual with Pl Pl platelets, who had never been transfused, was observed daily for 11 days after receiving 50 ml of 20-day old blood (containing Pl^{A1} Pl^{A1} platelets) from the initial donor of the full unit of blood to Patient 2. There was no evidence of antibody formation by C′ fixation, agglutination, and direct and indirect Coombs consumption techniques, and the above tests for "blocking" antibody were negative. A second injection of a suspension of 1.6×10^{11} fresh Pl^{A1} Pl^{A1} platelets, given to the same individual 11 days after the first injection, also did not produce a detectable form of anti-Pl^{A1} antibody based on determinations done on serum samples obtained daily for 2 weeks thereafter.

It was conceivable that individuals with Pl^{A1} Pl^{A1} platelets might lack a possible antigen "allelic" to Pl^{A1} and develop antibodies against it. Platelet-rich plasma from a normal *Pl Pl* individual was given intentionally to an individual with Pl^{A1} Pl^{A1} platelets. After administration of 125 ml on two separate occasions, the recipient did not develop antibody against Pl Pl platelets that could be detected with the same C′ fixation, agglutinin, and Coombs consumption techniques employed to measure the anti-Pl^{A1} antibody, and the recipient's platelet count was unaffected.

13. Evaluation of other relevant factors. In accounting for the infrequency of post-transfusion purpura, the possibility that a rare donor's blood might contain an unusual amount or form of antigen had to be considered. Attempts were made to detect a free or coating form of antigen in fresh samples of ACD blood obtained from the five donors whose blood had been given to both patients at operation; platelets from each donor reacted with the antibody, the donor of the full unit of blood given to Patient 2, and two of the three donors to Patient 1 having platelet type Pl^{A1} Pl^{A1}. Serum and plasma from each donor did not produce a visible band against antibody in agar-gel diffusion plates, and did not form a precipitin when layered on or mixed with serum containing 100 U of antibody per ml. Unheated, platelet-free sera from three of the five donors, used as

a source of possible antigen as well as C′ in incubation mixtures with antibody, did not fix C′; and prior incubation of small amounts of antibody (1.5 U) in 0.3 ml of the various donors' fresh platelet-free sera did not decrease the amount of C′ fixed by the antibody on subsequent addition of reactive platelets.[2] Thus, no evidence could be obtained for free Pl^{A1} antigen that would react directly with anti-Pl^{A1}. With the same series of tests for free antigen, none was detectable in Pl^{A1} Pl^{A1} blood after storage for up to 21 days at 5° C or after partial clotting was permitted before citrate anticoagulant was added. If age of blood or inadequate anticoagulant were involved in producing a free form of Pl^{A1}, the antigen was not detectable by the *in vitro* tests used.

Attempts to detect a soluble form of Pl^{A1} antigen in donor blood, which might be demonstrable by C′ fixation after adsorption on Pl Pl platelets, were unsuccessful, but two samples of plasma from the donor to Patient 2 contained large amounts of finely particulate antigen which did not sediment (see Section 6) unless Pl Pl platelets were mixed with the plasma. Pl Pl platelets, incubated in plasma of the donor to Patient 2 and washed, acquired the ability to fix C′ with antibody. However, filtration of the donor's plasma (300 mμ pore-size; see Section 6) removed the particulate antigen which apparently could be adsorbed by Pl Pl platelets.

Excretion of Pl^{A1} antigen in saliva could not be evaluated conclusively, for relatively undiluted saliva had marked anticomplementary activity. However, saliva from four individuals with Pl^{A1} Pl^{A1} platelets, including the donor to Patient 2, used at 1/10 dilution, did not fix C′ with antibody or interfere with complement fixation by Pl^{A1} Pl^{A1} platelets; and Pl Pl platelets, incubated in these undiluted salivas, did not acquire the ability to fix complement with antibody.

It was of interest to determine whether the Pl^{A1} antigen was present on platelets at birth. Platelets in cord blood of two infants were found to have the same antigen content as the Pl^{A1} Pl^{A1} platelets of the mothers.

14. Observations on typical cases of idiopathic thrombocytopenic purpura. None of 16 cases of classical ITP was found to have a platelet agglutinin, with a test system using sera at 8/10 or greater final dilution. Of the 16 cases, 5 were less than 15 years old; 4 of the 5 were males; 2 of the 5 had acute and 3 had chronic ITP. Eight of 11 adults were females; 3 of the 11 had acute and 8 had chronic ITP. Sera from 12 patients with ITP were used in C′-fixation tests with platelets from 6 different normal individuals, and in no instance was C′ fixed. Final platelet concentrations used in C′-fixation tests with ITP sera varied from 25,000 to 100,000 per mm^3, the final serum dilution was 1/2 to 1/4, and the amount of guinea pig C′ used permitted detecting with certainty fixation of 0.5 U. All C′-fixation mixtures were incubated for 90 minutes at 37° C. In addition, in three instances in which the ITP patient's own serum was used as a source of possible antibody as well as C′, C′ was not fixed. Thus, the various *in vitro* techniques, used at the limit of their sensitivity for detecting the anti-Pl^{A1} antibody and other definite platelet antibodies (8, 9), gave no evidence for the presence of a similar antibody in the sera of typical ITP cases.

Platelets from eight consecutive cases of ITP reacted with the antibody from Patient 1 in C′ fixation and agglutination tests in the same way as did platelets from normal individuals. Antigen content of these platelets was not determined quantitatively.

DISCUSSION

Development of the disease. The correlation in the two patients studied of transfusion of mismatched platelets, thrombocytopenia 1 week later, and the presence of an antibody which reacted with the donors' platelets, but not with the patients' own platelets obtained after recovery, indicates that an isoantibody was provoked which paradoxically was capable of destroying platelets of the sensitized individuals.

There are two published reports of patients with thrombocytopenic purpura associated with a platelet agglutinin (17, 18) and inhibtor of clot retraction (17) which did not react with the patients' own platelets obtained after recovery. Rea-

[2] When human serum instead of guinea pig serum was used as a source of C′, antigen-antibody mixtures fixed half as many units of human C′ as guinea pig C′. The decrease in C′ fixation was not due to specific inhibition produced by free Pl^{A1} antigen; for all human sera used as a source of C′, whether from individuals with Pl^{A1} Pl^{A1} or Pl Pl platelets, gave identical results in C′-fixation tests.

sons for development of the antibody and the thrombocytopenic state in these cases were not clearly defined. Actually, the two reported patients had received a blood transfusion shortly before onset of purpura and the clinical course of all four patients was strikingly similar (Table V). Serum from the patient of van Loghem, Dorfmeijer, van der Hart and Schreuder (18), had agglutinated platelets from 97.6 per cent of the normal individuals tested, and the antigen which reacted with the antibody was labeled "Zw." We sent antibody which reacted with antigen, that we have labeled Pl^{A1}, to Dr. van Loghem who found that it reacted with Zw-positive platelets, but not with Zw-negative platelets. The antigen Pl^{A1} is, therefore, the same as Zw, and the term, Zw, has precedence; but the designations Pl^{A1} and *Pl*, for the genetic determinants of platelet antigenicity, facilitate presentation of the concepts derived from the present study and are in accord with systematic notation suggested by Ford (21) (see below). No doubt three of the cases represent immunization of *Pl Pl* individuals against Pl^{A1} antigen, and there is little doubt that the patient of Zucker and co-workers (17) represents immunization against a platelet antigen but, unfortunately, the specificity could not be determined.

During the period of purpura, the patients' platelets, nonreactive on a genetic basis, evidently were susceptible to the antibody. This susceptibility was not due to some obscure reaction between antibody and normal Pl Pl platelets which might occur only *in vivo*, for 13 times the amount of antibody that produced sustained depression of platelets in a Pl^{A1} Pl^{A1} individual had no effect on platelets of a *Pl Pl* individual (Section 11). Since recovery took place in Patient 1 (Figure 3) in the presence of an antibody level much higher than the highest level measured in Patient 2 during purpura (Figure 1), it was apparent that beneficial effects of exchange transfusion were not due simply to decrease in antibody. Rather, the abrupt cure appeared to be caused by suppression of an additional factor which must have been present to make Pl Pl platelets susceptible to antibody during the period of thrombocytopenia. The observed phenomena could be explained if Pl^{A1} antigen in transfused blood not only provoked antibody, but also persisted *in vivo* in a form capable of effecting attachment of antibody to Pl Pl platelets. The following considerations lend weight to this hypothesis.

There are at least two ways in which antibody against Pl^{A1} antigen could become attached to Pl Pl platelets; these platelets could be coated *in vivo* with some form of antigen that would react with antibody, or antigen-antibody complexes formed *in vivo* could be adsorbed onto platelets. The first possibility is supported by well documented examples that free antigens are adsorbed on erythrocytes both *in vitro* and *in vivo*—e.g., Lewis Groups (22a) and the J antigen in cattle (23); but, as yet, there are no examples that transfused erythrocyte coating-antigens provoke antibodies which cause destruction of the recipient's erythrocytes. The second possibility is supported by observations which indicate that adsorption of antigen-antibody complexes onto platelets is the most likely mechanism of drug purpura (4, 8, 24), and that nonspecific adsorption of antigen-antibody complexes by platelets is a general immunologic phenomenon (25) which can produce thrombocytopenia *in vivo* (26). Either possibility would depend on survival of Pl^{A1} antigen *in vivo* for a longer time than the period of antibody induction.

Results of infusions of antibody in normal Pl^{A1} Pl^{A1} individuals (Figure 10) indicate the amount of Pl^{A1} antigen that would have to survive to cause thrombocytopenia by the above mechanisms. As little as 360 U of injected antibody, an amount which could saturate at most the 3.6×10^9 Pl^{A1} Pl^{A1} platelets (Figure 7) contained in approximately 20 ml of the recipient's blood, produced sustained depression of platelets in a 54 kg individual. By analogy, it appears that if Pl^{A1} antigen from as little as 20 ml of blood were distributed over the platelets of a *Pl Pl* recipient in association with antibody, thrombocytopenia would no doubt occur. A form of antigen that might coat Pl Pl platelets or be adsorbed onto platelets in complex with antibody would have to be detached from antigenic platelets. The *in vivo* survival of antigen which became detached from a donor's platelets (either before blood was transfused or by some *in vivo* process in the recipient) would not necessarily be comparable with survival of intact transfused platelets. Because the antigen is so stable (Section 6), and such small amounts apparently would suffice to make Pl Pl platelets susceptible to anti-

body *in vivo*, it is conceivable that, after transfusion of 500 ml of Pl^{A1} Pl^{A1} blood, an effective antigen concentration could persist for weeks *in vivo*.

Numerous attempts were made to demonstrate a form of antigen that might effect attachment of antibody to Pl Pl platelets (Sections 6 and 13), but none could be detected in blood from normal individuals, including three of the patients' initial donors, or in various preparations of disrupted platelets. It is possible that conditions for producing the appropriate form of antigen may exist only *in vivo*. The finding that Pl^{A1} antigen could be altered so that its complexes with antibody would not give the usual *in vitro* reactions (Section 6) suggests that a form of antigen, not necessarily detectable by techniques used so far, could be involved in development of the disease.

It is apparent from the various conditions which failed to dissociate antibody-Pl^{A1} antigen complexes (Section 7), and from the completeness of antibody adsorption in mixtures containing an approximately equal ratio of antigen and antibody (Figure 7), that the association constant of the complex must be very high. This favors the possibility that circulating antigen remaining at the time antibody appears exists thereafter in complex with antibody, and that the complex is adsorbed by Pl Pl platelets. The high affinity between antibody and antigen would assure complex formation even at very low concentration of both reactants and could account for persistence of thrombocytopenia under the conditions present in Patient G.H. Smaller amounts of antigen-antibody complex may be required to perpetuate the thrombocytopenic state than to establish it, for megakaryocytes apparently have a limited capacity to replenish platelets after induction of severe thrombocytopenia (4).

The above considerations, therefore, make it possible to explain the occurrence of post-transfusion purpura in terms of *in vivo* survival of minute amounts of foreign antigen which, in complex with the antibody it provokes, can attach to platelets of the recipient. Other conceivable explanations for the paradoxical thrombocytopenia would require such hypotheses as the presence of a different population of platelets before immunization in individuals not showing evidence of chimerism (27), the presence of a different population of platelets after the thrombocytopenic state as a result of somatic mutation, or the temporary presence of acquired Pl^{A1}-like substance on Pl Pl platelets analogous to acquired B-like substance on Group A erythrocytes (28). The likelihood that these hypothetical circumstances would associate by chance with immunization against Pl^{A1} antigen, and account for thrombocytopenia, seems more remote than the explanation discussed above.

The infrequency of post-transfusion purpura. The problem of explaining the infrequency of post-transfusion purpura, in spite of frequent antigenic mismatch (approximately 1.5 per cent of general transfusions), has two aspects. One aspect involves factors affecting development of thrombocytopenia if antibody does form; the other, factors affecting formation of antibodies against so-called "weak" antigens. Development of antibody alone without accompanying thrombocytopenia conceivably could occur, for antibody might be provoked in susceptible *Pl Pl* individuals by any form of Pl^{A1} antigen, but the form of antigen necessary to effect attachment of antibody to platelets may not develop or survive the period of antibody induction so frequently. The frequency with which antibody alone may develop is not known, because purpura is the only symptom that draws attention to immunization. Failure of attempts to provoke antibody in a normal *Pl Pl* individual (Section 12) established the fact that *Pl Pl* individuals do not always form detectable antibodies or develop thrombocytopenia after initial exposure to Pl^{A1} antigen. Alternatively, the form of antigen that may survive *in vivo* and effect attachment of antibody to Pl Pl platelets may be the only type that gains access to antibody-forming tissue. Failure of Patient 1 to respond to attempts at re-immunization (Section 12) suggests that some unique characteristic of antigen in donor blood may be a prerequisite for immunization. Otherwise, it would appear that Pl^{A1} antigen either could produce "immunologic paralysis" (29), a phenomenon which has not been observed in human beings as yet, or provoke another form of antibody undetectable by techniques used.

There are other possible factors which could affect initial "susceptibility" to transfused Pl^{A1} antigen. Some *Pl Pl* individuals may acquire immunologic tolerance (30) as a result of placental transfer of maternal Pl^{A1} antigen, since most

Pl Pl individuals would have Pl^{A1} *Pl* parents (see below). If placental transfer occurred invariably, only children of *Pl Pl* mothers might be expected to be sensitive to Pl^{A1} antigen; but the two patients observed had Pl^{A1} *Pl* mothers (Figure 4), indicating that, if immunologic tolerance does occur, it is not predictable. The two observed patients each had three Pl^{A1} *Pl* children, and the two other patients (Table V) also had had children. If the disease proves to be limited to parous females, conditioning to placental transfer of Pl^{A1} antigen present in fetal blood may have to be considered as a susceptibility factor. The meaning of the equivocal terms "weak antigen" and "susceptibility," applied in the case of platelet mismatch, may become clearer when these possibilities are further evaluated.

Therapy of the disease. Antibody production in this disease is typical of that following an initial single exposure to an antigen; for after the peak response, by the sixth to eighth post-transfusion day, there was a gradual decline in titer (Figure 1) (31). Thrombocytopenia in Patient 2 cleared concomitantly with decay of antibody, and in Patient 1, possibly after removal of the postulated antigen-antibody complex. The disease is therefore self-limited, and purpura apparently clears spontaneously when antibody or the postulated antigen-antibody complex, or both, reach a level low enough to permit regenerating platelets to circulate. However, since purpura is characteristically severe and lasts as long as 3 weeks, treatment may become necessary.

Exchange transfusion may prove to be the treatment of choice, for all evidence indicates that steroids are not beneficial (see Table V) and splenectomy does not seem to be indicated; but there is some hazard attending exchange transfusion. During the period of purpura, all patients had reactions to whole blood or platelet-rich plasma, such as chills, fever, urticaria and shock, all of which might be attributable to reaction between Pl^{A1} antigen on transfused platelets and antibody. The long time required to effect the exchange in Patient 1 was due to the inability to infuse whole blood at a rate faster than 2 to 3 ml per minute without causing severe hypotension. The severity or type of symptomatology other patients may develop when similarly treated cannot be predicted. Although Pl^{A1} antigen given during the exchange did not provoke further antibody or perpetuate thrombocytopenia, until further information is obtained, it must be considered that additional Pl^{A1} antigen potentially may prolong, rather than cure, the disease. Perhaps exchange transfusion with Pl Pl blood, or administration of large amounts of Pl Pl platelets alone, if available, would provide equally effective treatment and entail less risk.

The exchange transfusion involved alternate removal and replacement of 100- to 300-ml aliquots of blood until a total of 5,500 was withdrawn and 6,500 replaced. Assuming a fixed blood volume of 4,900 ml (see Section 2 and Figure 3) and that excess transfused blood elevated the hematocrit but did not dilute plasma components, the final dilution produced, calculated simply as a series of sequential dilutions, was 31 per cent (or 69 per cent effective). Since this value would vary no more than $\pm$ 1 per cent whether the alternate aliquots withdrawn and replaced were 10 per cent or 1 per cent of the initial blood volume, not less than approximately 31 per cent of the initial antibody should have been left as a result of dilution alone; but only 3 per cent could be measured (Figure 3). However, the 6,500 ml of transfused blood, containing approximately 200,000 platelets per mm^3 (assumed 74 per cent Pl^{A1} Pl^{A1}, 24 per cent Pl^{A1} Pl), was sufficient to bind approximately 1.14×10^5 U of antibody based on the data of Figure 5 (i.e., 10^8 Pl^{A1} Pl^{A1} and 2×10^8 Pl^{A1} Pl platelets bind 10 U of antibody). Of the 3.2×10^5 U of circulating antibody (3,200 ml plasma $\times$ 100 units per ml) present initially, apparently 69 per cent, or 2.2×10^5 U, was removed by exchange and 1.14×10^5 U could have been sequestered with the transfused platelets which did not circulate. Thus, combined removal and sequestration could account for loss of approximately as much antibody as circulated initially, and hence for the finding of 3 per cent antibody left. Within 13 hours after the exchange, circulating antibody had increased to 6.4×10^4 U (3,200 ml plasma $\times$ 20 U per ml). The elevation was too prompt and too moderate to be considered an anamnestic response and suggested that re-equilibration with extravascular γ-globulin occurred, which in part could have reflected some release of the 1.14×10^5 U of sequestered antibody.

Since approximately 2 U of human C′ (one-half

as much as guinea pig C′, Section 13) was fixed by 1 U of antibody, administration of enough antigen to complex with 1.14×10^5 U of antibody (see above) could have resulted in fixation of as much as 2.28×10^5 U of C′ *in vivo*. The patient's total circulating complement was initially 1.76×10^5 U (55 U C′ per ml × 3,200 ml plasma), and the difference between the 2.34×10^5 U of C′ in transfused plasma (an average of 60 U C′ per ml × 3,900 ml) and the 1.54×10^5 U of C′ in removed plasma (an average of 44 U C′ per ml × 3,500 ml) would have provided an additional 0.8×10^5 U of circulating C′. Thus, 2.56×10^5 total units of C′ were available and 2.28×10^5 (or approximately 90 per cent) could have been fixed. The measured drop of C′ *in vivo* was the result of fixation, for it was subsequently observed that exchange transfusion with even larger amounts of ACD blood in a nonimmunologic disease did not produce a drop in C′. The patient's C′ level fell as little as it did (Figure 3) possibly because additional amounts were produced rapidly or were available from extravascular spaces, or both. Because such large amounts of C′ may be fixed with relatively little effect on the circulating C′ level, it is not surprising that both patients had normal levels of C′ during the height of purpura, for the amounts of antigen conceivably involved in development of the disease were quite small.

Characteristics of the platelet system containing Pl^{A1} antigen. The ability to differentiate clearly three cell phenotypes based on quantitative reactions with an antibody against one antigen is unique. Since platelets of individuals who were most likely Pl^{A1} *Pl* uniformly contained half as much antigen as platelets of individuals who were most likely Pl^{A1} Pl^{A1}, it appears that the known antigen, Pl^{A1}, is inherited as a character, dependent on a gene capable of expressing itself in a single or double dose. In view of the dominance of blood group antigens in general (22b), there is a good possibility that the allele of Pl^{A1} controls another antigen which is inherited as a "co-dominant" character. Attempts to find or provoke an antibody against this hypothetical antigen (or antigens, for conceivably there could be more than one allele) have been unsuccessful so far (Sections 10 and 12). Since similar attempts to provoke antibody against the known Pl^{A1} antigen also failed, the possibility of finding the "missing" antigen(s) still seems more likely than the possibility that the *Pl* gene has no activity or only modifies (22c) the expression of Pl^{A1}.

The gene frequency of Pl^{A1} and *Pl*, estimated by translating low and high activity phenotypes into genotypes Pl^{A1} *Pl* and Pl^{A1} Pl^{A1}, respectively, was in close agreement with the frequency that would be required to give the observed 1.5 per cent nonreactors in the group of 206 individuals tested. Since there were 26 per cent apparently heterozygous and 74 per cent apparently homozygous Pl^{A1} individuals among the 23 individuals screened by quantitative C′ fixation, the gene frequency (22d) of Pl^{A1} would be $0.74 + 0.26/2 = 0.87$, and of *Pl*, $0.26/2 = 0.13$. The expected frequency of *Pl Pl* individuals on this basis would be $(0.13)^2 = 0.017$ or 1.7 per cent, which compares well with the observed frequency of 1.5 per cent in the large group.

Effects of various physical and chemical forms of treatment (Section 6) indicated that Pl^{A1} antigen could be either a relatively stable protein or polysaccharide, and the ratios of C′-fixation activity to nitrogen or sugar content of platelet fractions (Table II) did not distinguish the possibilities. However, since prolonged exposure to high concentration of trypsin had no effect on the antigen, there is a good possibility that Pl^{A1} is a polysaccharide, perhaps similar to the mucopolysaccharide erythrocyte group substances which have been characterized (32, 33). The Pl^{A1} antigen could not be detected by fluorescein-labeled antibody techniques (Section 8). In this respect Pl^{A1} antigen resembles A substance on erythrocytes (34) and differs from the human species-specific platelet antigens which provoke heterologous antibodies (12, 19) which do not distinguish type-specific human platelet antigens (Section 10).

By determining platelet genotypes and erythrocyte phenotypes in the same individuals, it was possible to conclude that Pl^{A1} antigen was not identical with a number of different erythrocyte antigens (Section 5), but more observations on appropriate family groups will be necessary to evaluate possible linkage between platelet and erythrocyte group systems. Since the platelet antigen system is another example of a genetic polymorphism (35), some correlation between this system and a factor influencing natural selection may exist. The selection factor conceivably could be

neonatal purpura (36) due to mother-fetal Pl^{A1} incompatibility; for we have recently seen a family in which the mother has type Pl Pl platelets, the father has type Pl^{A1} Pl^{A1}, and a child born a year ago with neonatal purpura has type Pl^{A1} Pl.

Definite demonstration of a direct proportionality between gene dose and quantity of antigen on platelets was made possible by use of a quantitative C′-fixation technique. Other serologic procedures used did not lend themselves to sufficient refinement for differentiating cells containing a twofold difference in antigen (Section 5). Over the years many attempts have been made to distinguish heterozygous erythrocyte phenotypes by agglutination techniques (22) and, more recently, by employing radioactively labeled antibodies (37, 38), but the results have not been clear-cut, possibly due to limitations of techniques used. It appears likely that application of a C′-fixation technique, similar to the one used herein, will facilitate more precise evaluation of erythrocyte antigen systems (and gene-dose effects) in the instances in which this technique is applicable.

Notation of platelet antigen systems. Because it is likely that at least one allele and possibly other alleles of Pl^{A1} will be identified, and because we have recently identified several other inherited platelet antigens [3] which are not produced by an allele of Pl^{A1}, some uniform notation of platelet antigen systems should be adopted. Many of the suggestions of Ford (21) have been followed in this report. Genes are italicized, antigen not italicized; the gene whose antigen has been recognized by an antibody is indicated by the suffix Al, and no suffix has been given to the gene whose antigen has not been recognized as yet. It is suggested that if an allele of Pl^{A1} is recognized by an antibody, it be labeled Pl^{A2}; and if multiple alleles are recognized, they be labeled sequentially, Pl^{A3}, and so on.

The matter of labeling loci of platelet antigen systems to distinguish them from erythrocyte systems can be overcome if new loci controlling platelet antigens are designated by a new suffix letter after *Pl*—i.e., Pl^{B}, Pl^{C}, and so on,[4] and if the alleles at each locus are identified by suffix numbers. A gene whose antigen has not been recognized by an antibody could be labeled with the appropriate suffix letter, but not a number. For example, the system reported herein would be called the Pl^{A} system; the recognized allele would be designated as it has been, Pl^{A1}, and the unrecognized allele would be Pl^{A} instead of *Pl*. This is a departure from Ford's suggestion but would provide an open end for any number of alleles in any one system; each system would have its distinguishing suffix letter, and there would be no confusion with erythrocyte or other systems. Because phenotypes can be translated directly into genotypes in the Pl^{A} system, antigen-dose and gene-dose could be indicated by numbers in brackets after the locus symbol, and absence of a "dose" could be indicated simply by a minus, i.e., Pl^{A} (1,1) for Pl^{A1} Pl^{A1} platelets, and Pl^{A} (1,1) for the corresponding genotype; Pl^{A} (1,-) for Pl^{A1} Pl^{A} platelets, and Pl^{A} (-,-) for Pl^{A} Pl^{A} platelets. The number, 2, would be substituted for a minus when the antigen is recognized. If only the presence or absence of an antigen were detectable in another platelet antigen system, only one number or minus would be placed in a bracket. The suggestions by Ford concerning recessive antigens could be followed by using a small suffix number.

Implications in ITP and suspected "autoimmune" disorders. Post-transfusion purpura is the second definite immunologic entity to be differentiated from the ITP syndrome. The first was drug purpura. Although the underlying causes of sensitization are widely different in post-transfusion and drug purpura, the antibodies in both diseases have similar qualitative and quantitative activities *in vitro* and *in vivo*. The similar relationships in the two antibody systems, between the amount of C′ fixed and the relative concentrations of antibody and platelets (see Figure 8 and Reference 8) indicate that the theoretical considerations concerning antibody adsorption and C′ fixation which apply to drug antibodies (8, 39) also are applicable to anti-Pl^{A1} (as well as to heterologous antibodies; e.g., Figure 9) if variables de-

[3] During preparation of this report, the mother of an infant with neonatal purpura was found to have an antibody in her serum which reacts with an antigen inherited independently of Pl^{A1}. An abstract concerning this finding appeared in the April, 1961, issue of Clinical Research. Moreover, antibodies against two additional platelet antigens, each apparently genetically unrelated to the other three, have been found in individuals who had received multiple transfusions.

[4] The additional loci, Pl^{B}, Pl^{C}, and Pl^{D} are already accounted for in this laboratory (Footnote 3).

pendent on drug concentration are excluded. Thus, the mechanism proposed for C′ fixation by drug antibodies (8) may operate generally with many antibodies reacting with antigens on cellular surfaces.

The concentration of anti-Pl^{A1} required to depress Pl^{A1} Pl^{A1} platelets experimentally *in vivo* was much lower than the concentration that could be detected *in vitro* with the most sensitive assay techniques developed so far (Figure 10). Platelets which were destroyed *in vivo* were quite unsaturated with antibody. The degree of unsaturation was such that platelets probably were not "lysed" directly by the small amount of C′ which could have been fixed and probably were not agglutinated in the circulation. Rather, platelets with minimal amounts of antibody attached apparently were rendered more susceptible to processes of *in vivo* sequestration, just as in the case of thrombocytopenia due to the antibody of quinidine purpura (4). The observations that dogs are resistant to *in vivo* effects of anti-Pl^{A1}, and that dog C′ is not fixed as effectively as human C′ by this antibody system (Sections 9 and 11), indicate that C′ fixation may play a role in sequestration of antibody-coated cells.

The fact that a C′-fixing antibody is not demonstrable in the usual case of idiopathic thrombocytopenic purpura, by techniques used so far, does not conflict with the possibility that ITP is an immune disease. Analogies drawn from a study of quinidine purpura (4, 8, 9) made it possible to explain the characteristics of ITP in terms of immunoreactions occurring at low levels of activity which could not be detected by usual *in vitro* techniques, and similar analogies can be drawn from the present study of post-transfusion purpura. Since drug purpura and post-transfusion purpura are due to immunization against foreign antigens and the mechanism of thrombocytopenia in both diseases appears to be similar, there is reason to believe that a variety of other foreign antigens may be identified as the underlying cause of a group of specific disorders falling within the syndrome of ITP.

The ITP syndrome resembles a variety of other diseases in which an individual's own cells are injured or destroyed by apparent immune processes (2, 40). Demonstration that the manifestations of ITP can be duplicated by antibodies provoked by foreign antigens suggests that sensitization by foreign antigens may underlie other so-called "autoimmune" disorders.

SUMMARY

1. The clinical picture of post-transfusion purpura and a successful method of treatment was presented.

2. An isoantibody provoked by a mismatched platelet antigen destroyed platelets in the sensitized individuals. It is proposed, as a mechanism of thrombocytopenia, that foreign antigen survives *in vivo* longer than the period of antibody induction and that antibody, complexed with foreign antigen, is adsorbed by autologous platelets.

3. Infrequency of purpura, despite frequent mismatch of platelet antigens in general transfusions, may be accounted for by various factors that affect development of antibody as well as survival of antigen *in vivo*.

4. Experimental infusions of antibody indicate that thrombocytopenia can occur when *in vivo* concentrations of antibody are below levels detectable by currently available *in vitro* tests. Minimal amounts of antibody and complement attached to platelets appear to increase their susceptibility to sequestration.

5. Similarities which were found between the immunoreactions of post-transfusion and drug purpura permit interpreting the pathogenesis of idiopathic thrombocytopenic purpura (and possibly other disorders thought to be "autoimmune") in terms of sensitization by foreign antigens.

6. The antigen responsible for the disorder is inherited as a character dependent on a gene capable of expressing itself in a single or double dose. A clear-cut proportionality between gene dose and quantity of antigen per cell was demonstrated.

7. A uniform notation for platelet antigen systems is proposed.

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ENDOGENOUS PRODUCTION OF FIXED ACID AND THE MEASUREMENT OF THE NET BALANCE OF ACID IN NORMAL SUBJECTS *

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It is commonly assumed that one of the essential functions of the kidney is to maintain acid-base balance by excreting an amount of acid equivalent to the net quantity of protons released from fixed (i.e., nonvolatile) endogenous acids. Excretion of acid in the urine is easily measured. Therefore, if the net rate of production of fixed acids could also be measured independently, it might be possible to evaluate the external acid balance of the body in the same manner as the balance of mineral elements or nitrogen. In the normal steady state, the net excretion of acid in the urine (titratable acid plus ammonium, minus bicarbonate) would be expected to equal the sum of the nonmetabolized acids ingested plus those endogenously produced. Except for clinical situations in which stool losses of alkali or acid might also require consideration, all metabolic disturbances of the net acid-base balance of the body would thus be quantitatively determined by the difference between the acid ingested or endogenously produced and that excreted in the urine.

At present, however, there is no clear definition of all the sources of endogenous fixed acids nor does there exist any satisfactory method for accurately quantitating the net acid load independently of measurements of renal acid excretion. It is sometimes assumed that it is the calculated "acidity" or "alkalinity" of the diet "ash" which normally determines the endogenous load of fixed acids. This calculation, based simply on the discrepancy between mineral cations and the chloride, phosphorus and sulfur of the diet, has theoretical as well as practical defects (1–4), and does not in fact correlate well with the rate of acid excretion (5).

Hunt has recently adduced evidence to support his suggestion that acid derived from the oxidation of organic sulfur to sulfate constitutes the major, if not the sole, source of fixed acid (5). In subsequent experiments from this laboratory (6) a detailed study was made of the acidifying effects of large oral methionine loads. It was found that the increased oxidation of organic sulfur to sulfate was accompanied by the systemic release of approximately equivalent quantities of protons, which ultimately were excreted in the urine. In many of Hunt's experiments, however, net acid excretion in the steady state was significantly greater than the measured excretion of sulfate. In our own studies, in which a natural soy flour, rich in potassium, was employed as the nitrogen source, net excretion of acid in the steady state prior to loading with methionine always was found to be approximately 20 to 30 per cent *less than* the excretion of sulfate (6). It thus appeared that the net endogenous production of fixed acid could not be equated solely with sulfate production.

The present study was therefore designed to investigate the normal sources of fixed acid more thoroughly. A new liquid-formula diet was devised which utilized a purified soy phosphoprotein virtually free of mineral cations. This made it possible to measure the acid contribution from the dietary protein more precisely. It was then found that the discrepancy between sulfate production and the excretion of net acid in the steady state could be quantitatively accounted for by the sum of the total organic acids in the urine plus the acid derived from the combustible cations neutralizing the phosphate groups in the protein fed. It was therefore concluded that the net production of endogenous fixed acids with the present diet was determined by the sum of: *a*) the oxidation of organic sulfur in the fed protein, *b*) the liberation of excess protons from the cations covering the

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phosphate radicals in the protein, and *c*) the endogenous formation of unmetabolized organic acids. The existence of an apparent physiologic balance between acid excretion and the production of fixed acid measured in this way was also demonstrated in a few relatively prolonged balance studies in which predictable changes in acid balance were temporarily induced by experimental alteration of the excretion or net production of acid.

METHODS AND MATERIALS

Ten balance studies were performed on 9 healthy young adult males. Liquid-formula diets were used which provided 35 to 40 calories and 0.8 to 1.0 g protein per kg body weight per day. Corn oil, glucose and dextrin served as sources of fat and carbohydrate. A pure soybean protein was used as the protein source. According to the manufacturer, this protein[1] is essentially uniform by electrophoresis. It is virtually free of minerals other than phosphorus as shown by the following analyses: sodium, 1 mEq/100 g; potassium, 3 mEq/100 g; calcium, < 0.1 mEq/100 g; magnesium,[2] < 0.1 mEq/100 g; chloride, < 1 mEq/100 g; phosphorus, 17 mmoles/100 g (0.53 per cent).

This protein was prepared at its isoelectric pH (5.0 to 5.2) and does not disperse well in water. In 7 balances L-arginine (free-base), in quantities ranging from 25 to 57 mmoles per day (0.46 to 0.64 mmoles per g protein), was used to raise the pH of the diet mixture to approximately 7.0 and thus effect dispersion of the protein. In one subject small amounts of ammonium hydroxide were used instead of arginine. In the remaining 2 balances the protein was dispersed by the addition of $Ca(OH)_2$ and $Mg(OH)_2$, as described in the following paragraph.

In all of the studies except those two in which $Ca(OH)_2$ and $Mg(OH)_2$ were given, the calculated acid derived from the phosphoprotein was added to the acid production measured as urinary inorganic sulfate and organic acids. This calculation was made on the assumption that the phosphate esters present in the protein were completely hydrolzed and the covering cations completely metabolized. Metabolism of an organic monophosphate ester does not necessarily yield protons. This result will depend upon the nature of the cationic group neutralizing the charge on the phosphate residues. At the isoelectric pH of the protein, phosphate groups are neutralized by protons or cationic amino groups. When arginine free-base or NH_4OH is used to raise the pH of the isoelectric protein (as was the case in 8 of the present balance studies), the cations neutralizing the phosphate charges are combustible and hence metabolism of these cations will ultimately yield excess protons, just as if the isoelectric protein had been fed. In either case, the number of protons released can be calculated by knowing the number of phosphate residues present, the pK' of the phosphate and the final pH of the medium. Thus, with reference to the excellular pH (7.40), each mole of phosphorus fed would be expected to yield 1.8 equivalents of acid (pK_2' of phosphoric acid $= 6.8$). If $Ca(OH)_2$ or $Mg(OH)_2$ is used to raise the pH of the protein, the cation "covering" the phosphate (Mg^{++} or Ca^{++}) is not combustible, and hence no protons will be released. In the two experiments in which $Ca(OH)_2$ and $Mg(OH)_2$ were given instead of arginine, the amount of alkali added was equal to the calculated excess of H^+ which is released if a combustible alkali such as ammonium hydroxide or arginine free-base is used to disperse the protein. Under these circumstances, therefore, the acidifying effect of the arginine phosphate or ammonium phosphate is avoided, and the hydrolysis and metabolism of the phosphate residues contribute no endogenous acid. It should be noted that, when arginine or ammonium phosphate is fed, it is not the phosphate residues but rather the combustible cations neutralizing these residues which give rise to the acid. Nevertheless, the *quantity* of acid so released can be calculated only from a consideration of the number of phosphate residues in the protein fed. (Any arginine or ammonia added to the phosphoprotein in excess of this number of residues does not give rise to acid.) Any noncombustible anionic residue on the protein would play the same role here as the phosphate, except that the pK of the residue determines what proportion of the protons yielded by metabolism of the combustible cations will continue to be held by the anionic residue at body fluid pH and what proportion will be given up to be handled by the physiological mechanisms for acid excretion.

The soybean protein contained adequate amounts of all the essential amino acids except methionine, so that a supplement of 1 g of DL- or L-methionine was provided daily in the basic diets. In addition, 50 to 80 mEq of potassium chloride and 90 to 200 mEq of sodium chloride per day were added to the diets. In 2 experiments 15 mmoles per day of calcium phosphate (as $CaHPO_4$) was also added. In these 2 studies correction was made for the alkali thus fed, arbitrarily assuming virtually complete absorption. Each subject took 2 multiple vitamin capsules daily and, to prevent diarrhea, they also were given small doses of deodorized tincture of opium. All stools were formed, but soft in consistency.

Each study was preceded by a 2-day period of adaptation to the standard diet. The balance then began with a control period lasting 3 to 8 days. In 6 experiments only these control observations were made. In the other 4 studies the control period was followed by an experimental period lasting 5 to 7 days during which the following changes in the control regimen were made: *a*) 1 subject received sodium bicarbonate, 70 mEq per day in divided doses, in addition to the control diet; *b*) 2 subjects received oral loads of neutral (pH 7.40) sodium orthophosphate, the phosphate mixture replacing a part of the chloride in the control diet, with sodium intake being kept at the control level; and *c*) 1 subject was given acetazolamide, 1.0 g daily in divided doses, in addi-

[1] "J-protein-100" kindly made available by the J. R. Short Milling Co., Chicago, Ill.

[2] Magnesium analyses through the courtesy of Dr. W. Wacker, Peter Bent Brigham Hospital.

TABLE I

Experimental data *

Subject TW Day	Body weight	Blood pH	Serum Total CO_2	Serum Na	Serum K	Serum Cl	Urine Vol	Urine pH	Urine Total CO_2	Urine TA†	Urine NH_4	Urine "Net acid"‡	Urine Inorg SO_4	Urine Org§ acids	Urine Na	Urine K	Urine Ca	Urine Cl	Urine P	Stool Na	Stool K	Stool Cl	Stool Ca	Stool P
	kg		*mmoles/L*	*mEq/L*			*ml/day*		*mmoles/day*	*mEq/day*									*mmoles/day*	*mEq/day*				*mmoles/day*
1	86.80						1,180	5.55		17	53	70	25	43	156	74	1.3	215	7	2.8	5.1	0.7	14	9
2	86.85	7.38	28.0	142	3.9	109	1,316	5.36		19	60	80	20	36	168	50	2.2	221	10	2.8	5.1	0.7	14	9
3	87.20						1,113	5.37		22	59	81	21	34	154	64	1.0	210	14	2.8	5.1	0.7	14	9
4	87.76	7.35	30.0	143	3.9	106	2,340	5.66		16	58	74	22	47	239	62	2.6	302	7	2.8	5.1	0.7	14	9
5	87.10		29.3	143	3.9	107	1,428	5.45		18	46	65	15	26	154	49	1.3	201	10	2.8	5.1	0.7	14	9
6	86.67	7.40	28.5	141	3.9	109	1,440	5.46		20	57	78	22	36	160	73	1.3	224	11	2.8	5.1	0.7	14	9
7	86.87						2,760	6.04	11.3	18	49	62	21	43	295	51	4.4	312	13	2.8	5.1	0.7	14	9
8‖	85.98	7.38	30.9	141	3.8	106	2,030	5.78		62	52	115	20	46	159	72	0.8	138	73	4.9	4.9	0.8	6	7
9‖	86.11	7.40	29.8	143	3.4	106	1,270	6.16	7.2	70	31	98	20	38	151	60	0.3	47	105	4.9	4.9	0.8	6	7
10‖	86.33	7.37	32.4	144	3.8	102	1,610	6.43	15.5	63	25	77	20	36	198	44	0.5	39	112	4.9	4.9	0.8	6	7
11‖	86.46		33.2	144	3.6	104	1,620	6.15	11.0	72	32	99	19	38	162	41	0.6	32	112	4.9	4.9	0.8	6	7
12‖	86.61						1,820	6.35	23.3	63	29	77	20	40	200	39	0.7	44	109	4.9	4.9	0.8	6	7
13	86.40	7.37	32.6	144	3.5	104	1,010	6.24	8.0	29	32	56	23	36	169	24	3.1	109	43	1.5	3.2	0.2	6	4
14	86.81	7.38	30.5	143	3.9	106	2,475	6.19	17.8	18	44	52	25	48	371	48	8.2	364	16	1.5	3.2	0.2	6	4
15	86.04		29.1	147	4.1	108	1,690	5.89	7.9	21	57	75	29	55	338	70	6.2	365	17	1.5	3.2	0.2	6	4
16	86.25	7.37	27.5	145	3.9	108	1,232	5.35		26	69	95	26	50	222	69	5.8	292	14	1.5	3.2	0.2	6	4
17	86.35						1,717	5.43		30	58	88	29	51	266	88	3.1	328	17	1.5	3.2	0.2	6	4
18	86.05	7.35	30.3	143	4.0	108																		

* Basal intake per day: Na 206 mEq, Cl 256 mEq, K 60 mEq, Ca 2.1 mEq, P 11 mmoles.
† Titratable acidity.
‡ $NH_4^+ + TA - HCO_3^-$.
§ Total organic acids corrected for creatinine.
‖ Neutral sodium orthophosphate, 105 mmoles/day, substituted for equivalent amount of NaCl in diet. Intake per day: Na 203 mEq, Cl 57 mEq, K 61 mEq, Ca 2.0 mEq, P 118 mmoles.

tion to the control diet. In each of these 4 studies the experimental periods were followed by recovery periods of 4 to 6 days' duration.

Further details of the balance technique in use in this laboratory, as well as descriptions of most of the methods used for the analyses of diets, urine, feces and blood have previously been reported (6, 7). In the present experiments "organic acids" in urine were measured by the Van Slyke and Palmer method (8), modified as follows. Phosphate was removed by mechanical shaking for 30 minutes with added calcium hydroxide powder. Remaining carbonate was then eliminated by addition of HCl until the pH was exactly at 2.70. A Cambridge model R pH meter with dipping electrodes and a constant temperature bath at 37°C was employed for this purpose. "Organic acids" were then determined by titrating from pH 2.70 to pH 7.40, with 0.1 N NaOH. Correction was made for creatinine. Additional techniques, used in a few studies, included: *a*) calcium, by the method of Clark and Collip (9) and *b*) citrate, by a pentabromacetone method (10).

RESULTS

I. Control observations during the steady state (ten subjects)

A. Experimental data. Electrolyte balances were carried out in ten subjects under steady state conditions. In one, this period lasted only 3 days, and in another 5 days, but in the others the control observations lasted 6 to 8 days. Complete analytical data from one experiment which began with a control period of 7 days are shown in Table I. In this study, as well as in the other nine, serum electrolyte concentrations and CO_2 content and blood pH were essentially constant during the control periods. As shown in Table I, the urine pH and the daily excretion of all urinary constituents were also relatively steady. Fecal excretion of sodium, potassium and chloride was low. Fecal calcium varied in six experiments from 10 to 27 mEq per day and fecal phosphorus ranged from 6 to 11 mmoles per day.

Cumulative changes in weight were small and variable (−1.02 to +0.59 kg). Small positive cumulative sodium and chloride balances were noted in all subjects, consistent with the expected skin losses. Although not shown in the experiment of Table I, nitrogen balances were carried out in three subjects. Cumulative nitrogen balances ranged from −1.5 to +3.3 g. Cumulative potassium balances, uncorrected for nitrogen, varied from −13 to −109 mEq. Cumulative calcium balance varied from −111 to +6 mEq, and cumulative phosphorus balance ranged from −60 to +2 mmoles, the large negative balances resulting from stool losses in the subjects whose diets did not contain added calcium and phosphorus.

B. Derived data. The daily net excretion of acid (calculated as titratable acid plus ammonium minus bicarbonate) was approximately constant in every subject, as exemplified by the data in Table I, in the column headed "net acid." The excretion of inorganic sulfate was always less than half the net acid excretion, but it was noted that in each case the discrepancy between sulfate and acid excretion was approximately equal to the total "organic acid" excretion plus the calculated acid derived from dietary protein phosphorus. In each subject there were significant daily fluctuations in this relationship, as illustrated by the data in Table I, but on a cumulative basis the excretion of net acid for the entire control period was always very nearly equal to the sum of these three quantities. Table II presents a comparison of the cumulative excretion of net acid with the sum of excreted sulfate plus "organic acids" plus the calculated acid derived from the cation covering the phosphate residues in the protein. This comparison is made for the control periods only, in each of the ten subjects. The close agreement between acid excretion and production is apparent, the mean discrepancy being −0.4 ± 8.9 per cent. This result is also illustrated in the data in three individual subjects shown in the left-hand sections of Figures 1–3. An explanation of the design of these Figures is given below.

TABLE II

Comparison of net acid excretion (A) with the sum of urine sulfate and "organic acids," plus calculated acid phosphorus from diet (B) during steady state control conditions

Subject	Period	A	B	Discrepancy $\left(\frac{A-B}{B}\times 100\right)$
	days			%
TW	7	510	552	−7.6
RS	6	548	629	−12.9
JL	6	460	518	−11.2
JB	5	350	342	+2.3
GdeF	8	536	566	−5.3
RH	3	328	344	−4.7
FR	6	466	416	+12.0
PVanO, 1	6	591	556	+6.3
LS*	8	540	493	+9.5
PVanO, 2*	8	633	589	+7.5
Mean ± SD		496 ± 98	500 ± 101	−0.4 ± 8.9

* Dietary phosphorus neutralized by $Ca(OH)_2$ and $Mg(OH)_2$, hence not included in calculation of acid production (column B).

II. Effects of experimental changes in net acid production or acid excretion

If we assume the existence of a steady state of acid-base balance during the control periods, the data of Table II seem to imply that the only significant sources of endogenous fixed acid in these experiments were the protons released during: *a*) the oxidation of organic sulfur to sulfate, *b*) the generation of organic acids, and *c*) the combustion of the disposable cations covering phosphate residues in the dietary protein. In an attempt to determine whether acid-base equilibrium measured in this way could be demonstrated over even longer periods, and to ascertain whether induced changes in net acid production or excretion would have quantitatively predictable effects on this equilibrium, four additional experiments were carried out.

A. Sodium bicarbonate. In the first such experiment, an amount of sodium bicarbonate was administered which was equal to the estimated rate of acid production during the control period. The essential observations made in this study are summarized in Figure 1. In the upper half of the figure are shown the values for serum CO_2 content and 24-hour urine pH. In the lower half is shown the "acid" balance, plotted in the conventional manner of an electrolyte or nitrogen balance. Acid production, calculated in the manner already described, is plotted as a heavy line measured downward from the zero axis. Net acid excretion is measured upward from the line indicating acid production (diagonally-shaded area). Thus, clear areas below the zero axis represent positive proton balance, and extensions of the shaded area above the zero axis define a negative proton balance.

During the control period of this study, the average acid production and excretion was approximately 70 mEq per day and, as shown in the figure, net proton balance was virtually zero. Serum CO_2 content and blood pH were essentially constant, as was daily urine pH. After this, 70 mEq of $NaHCO_3$ was added to the daily diet for 7 days, as shown by the stippled area in the figure. Since the measured production of acid and the ingestion of protein phosphate remained virtually constant throughout the loading period, as shown by the dashed line, the *net effective* acid production was zero during this time. This is indicated in the figure by the heavy line which is nearly superimposed upon the zero axis. Acid excretion is plotted upward from this heavy line. During the first 3 days cumulated net proton balance was −85 mEq, because acid excretion did not cease immediately. Thereafter, acid excretion was essentially zero during the last 4 days of the experimental period. The reduction in net acid excretion was mainly accounted for by reductions in the excretion of ammonium and titratable acid. Although urine pH rose to approximately 6.6, no more than 28 mEq per day of the administered bicarbonate load was excreted as such in the urine. Serum CO_2 content rose slightly.

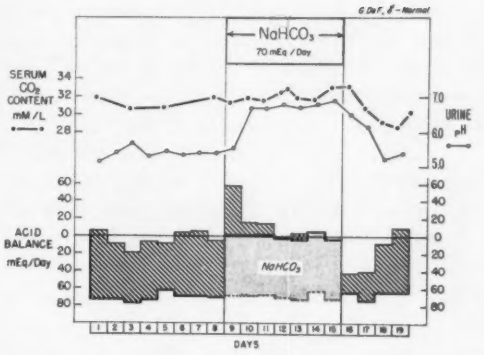

FIG. 1. NET EXTERNAL ACID BALANCE, SERUM CO_2 CONTENT AND URINE pH IN A NORMAL SUBJECT GIVEN $NaHCO_3$ FOR 7 DAYS. In this and subsequent figures, acid production is plotted downward from the zero line and net acid excretion in the urine is plotted as a diagonally-shaded column back up from the heavy line indicating acid production. Thus, a clear area *below* the zero line indicates a positive acid balance, and extension of the shaded area above the zero line indicates a negative acid balance. During the experimental period acid production is indicated by the dashed line but, due to the administration of $NaHCO_3$, *net* acid production is 70 mEq per day less than this, as shown by the heavy solid line. Urine acid excretion is plotted upward from this line.

When the administration of sodium bicarbonate was stopped, the net effective acid production immediately returned to control, but the acid excretion remained low for 2 more days before returning to the control rate. During this period the net retention of protons was 78 mEq, an amount closely approximating the net loss of 85 mEq of acid which had occurred at the beginning of the period of alkali administration. At the end

of 19 days, when serum CO_2 content and urine pH had returned to control, the total cumulative production of fixed acid (corrected for the bicarbonate fed) was 827 mEq, as compared with a cumulative net acid excretion of 806 mEq.

B. Neutral orthophosphate. The purpose of these two experiments was to increase acid excretion without changing acid production. This was accomplished by the administration of extra urinary buffer in the form of neutral inorganic phosphate. If measured acid production were to remain constant, this should result in a negative proton balance and the development of metabolic alkalosis. Furthermore, we would expect restoration of acid-base balance after cessation of phosphate loading to be accompanied by a demonstrably positive proton balance of approximately equal magnitude.

Subject TW (Table I) and Subject P VanO (No. 1, Figure 2) were given oral loads of neutral (pH 7.40) sodium orthophosphate for 5 days, the phosphate replacing part of the chloride in the control intake and the sodium intake remaining constant. After control periods, in which acid production and excretion in both studies were approximately equal, the phosphate load resulted in an immediate increase in net acid excretion. The rise in acid excretion was due entirely to increased titratable acid (Table I), which occurred despite a rise in urine pH and a reduction in ammonium excretion.

Since there was no significant change in calculated acid production, the initially increased excretion of net acid resulted in a negative acid balance. Cumulative balance of acid during the loading period was −66 mEq in TW and −112 mEq in P VanO. As would be predicted, there was a rise in serum CO_2 content in both cases, the final level being approximately 3 mmoles per L above control in TW and 5 above control in P VanO.

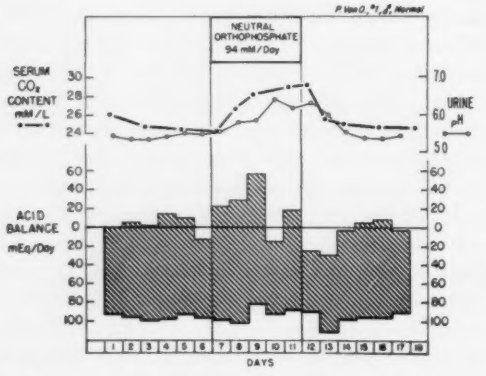

FIG. 2. THE EFFECT OF FEEDING NEUTRAL SODIUM PHOSPHATE ON NET EXTERNAL ACID BALANCE, SERUM CO_2 CONTENT AND URINE pH.

When phosphate loading was stopped, urinary excretion of phosphate immediately returned to control. For the first 2 days of the recovery period urine pH remained somewhat elevated and ammonium excretion was slightly reduced. Therefore, net acid excretion dropped sharply and acid balance became positive. Cumulative retention of acid during the recovery period was +106 mEq in TW and +48 mEq in P VanO. Serum CO_2 returned to control values and approximate equilibrium between production and excretion of acid was ultimately restored. For the entire period of study, cumulated acid production in TW was 1,421 mEq and cumulated excretion was 1,342 mEq. In the other subject (Figure 2) total production was 1,618 mEq and total excretion 1,693.

C. Acetazolamide. This experiment was designed to reduce renal excretion of acid without altering acid production. After a control period, the subject was given 250 mg of acetazolamide four times daily by mouth for 7 days. Administration of the drug produced a large diuresis of bicarbonate, and therefore net acid excretion was negative on the first day (Figure 3). This is illustrated in the figure by the dotted bar extending in a downward, or negative, direction from the level of acid production. Net retention of acid on this day was +226 mEq, and during this time serum CO_2 content fell from 31 to 24 mmoles per L. With continuing administration of drug, bicarbonate diuresis waned, and net excretion of acid became positive. However, acid balance continued to be slightly positive for the next few days and serum CO_2 content continued to fall slightly, reaching a minimal level of 21 mmoles per L. During the last 2 days of treatment the average acid balance was virtually zero and there was no further fall in serum CO_2 content.

On cessation of drug, excretion of net acid increased and exceeded acid production. Thus, during the last 4 days, acid balance was markedly

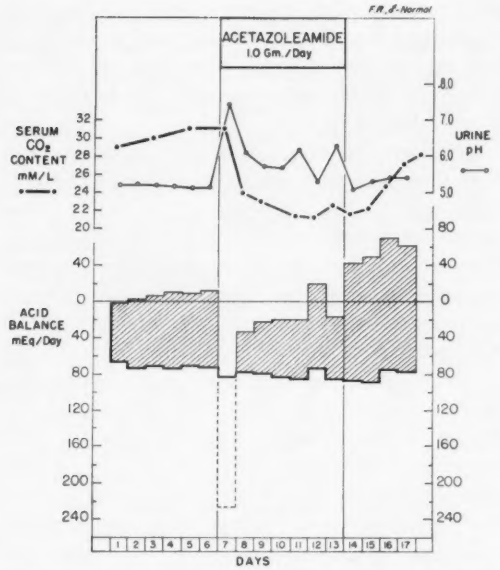

FIG. 3. THE EFFECT OF ADMINISTRATION OF ACETAZOLAMIDE ON NET EXTERNAL ACID BALANCE, SERUM CO_2 CONTENT AND URINE pH. On the first day of drug administration net acid excretion was negative, due to the loss of urinary bicarbonate. This is plotted as a dashed line downward from the heavy solid line indicating acid production. The net positive acid balance on that day is represented by the total distance between the dashed line and the baseline.

negative and serum CO_2 content was rising steadily.

On the fifth day of recovery the subject developed a gastroenteritis, and the study had to be terminated before the control state of acid-base equilibrium could be restored. At this time the cumulative acid production for the total balance was 1,340 mEq, as compared with a cumulative acid excretion of 1,259 mEq. The net balance of acid was therefore +81 mEq; the final serum CO_2 content was 28.2, as compared with the two final control values of 31 mmoles per L.

DISCUSSION

Most authorities assume that endogenous acid production results largely from the oxidation of organic sulfur- and phosphorus-containing compounds to inorganic sulfate and phosphate (11, 12), but until the recent study by Hunt (5) there had been no attempts at quantitative verification of any part of this hypothesis. Using various types of natural diets, Hunt found that net excretion of acid could be mainly accounted for by inorganic sulfate but observed no correlation between acid excretion and dietary phosphorus. He therefore suggested that endogenous acids arose almost entirely from the oxidation of sulfur; however, in several of his experiments sulfate was significantly less than net acid excretion. This discrepancy would have been even greater had the determination of "titratable acidity" been carried to the usual endpoint of pH 7.4 instead of to pH 7.0. A significant fraction of acid production therefore remained to be identified.

Recent experiments in this laboratory (6) have provided more direct quantitative evidence for the relationship of the oxidation of organic sulfur to fixed acid production. The results differed from those of Hunt in revealing the excretion of sulfate in the steady state to be significantly *greater* than the net excretion of acid. The explanation probably resided in the fact that the natural soybean flour used in those initial experiments contained potential alkali in the form of potassium, calcium and magnesium salts of the carboxyl groups in the protein. Since virtually all natural foods can be expected to contain variable and probably indeterminate quantities of "hidden alkali" in the form of such salts of metabolizable anions, it was decided to restudy the problem in the experiments reported here, with a synthetic diet and a protein source essentially free of fixed cation.

The present results indicate that less than half of net acid excretion in the steady state was due to oxidation of sulfur. The discrepancy could be almost quantitatively accounted for by the total organic acids in the urine, plus the calculated acid derived from the protein phosphorus in the diet. This seems to imply that under these experimental conditions the only significant sources of endogenous fixed acid were the protons released during: *a*) the oxidation of organic sulfur to sulfate (6), *b*) the generation of nonmetabolized organic acids, and *c*) the combustion of the disposable cation neutralizing phosphate residues in the dietary protein.

In the classical descriptions of the "acid-base" balance of the urine, organic acid anions were considered to represent "acid," but only in the sense that all anions were defined as "acids" and all cations as "bases" (13, 14). Such an approach

does not permit calculation of the actual net rate of fixed acid production and cannot logically be applied to a description of the acid balance of the body (3, 4, 15). It is well recognized that in certain abnormal states, such as diabetic ketosis, endogenous production of organic acids constitutes a major source of the acid load. In the present experiments, however, the special significance of urinary organic acids as one of the major sources of normal endogenous release of protons is clearly demonstrated. It seems reasonable that they should have this significance, for the organic acid anions normally appearing in the urine must represent acids produced during metabolism, which escape complete oxidation to CO_2 and H_2O and which therefore must have donated their protons to body buffers.

It should be noted that the relatively small fraction of excreted organic acids which appears in the urine as free acid (i.e., that fraction which is titrated between the pH of urine and the pH of plasma) does not contribute its protons to the body, and properly should be subtracted from the determination of "organic acids" in the calculation of acid production; but, since this free acid fraction is *also* measured in the determination of titratable acidity, net acid excretion by the kidney is overestimated by the same amount, and thus the calculation of net proton balance is unaffected.

The Van Slyke and Palmer titration is not a precise method and certainly does not measure organic acids alone (8). It includes not only creatinine (which must be subtracted from the total measured value) but also small quantities of amino acids and sulfate. The apparent pK for the carboxyl groups of the various amino acids ranges from approximately 1.8 to 2.4. Assuming normal urinary excretion of amino acids (up to 15 mmoles), partial titration of these carboxyl groups might overestimate organic acids by as much as 5 mEq per day. Since the apparent pK_2 for sulfuric acid is approximately 1.7, some 10 per cent of the urinary sulfate would also be titrated between 2.7 and 7.4, and in the present studies this would tend to overestimate organic acids by another 2 to 4 mEq per day. On the other hand, it has been reported (and we have confirmed) that nearly half of the urinary citrate may be lost during the "organic acid" titration, as a result of precipitation with calcium (16). During two of the present studies urinary citrate was found to range from 5 to 8 mEq per day, and it can therefore be estimated that the Van Slyke and Palmer titration probably underestimated citrate by about 2 to 4 mEq per day.

Considered together, all of these small errors in both directions tend approximately to cancel each other out. Although there are probably other sources of minor error in the method which have not been discussed, it appears that under the present conditions the Van Slyke and Palmer titration provides an approximate but reasonably satisfactory estimate of the contribution of organic acids to the generation of fixed acid. It is worth noting in this context that when the diet consists of natural foods containing organic acids the relationship of these compounds to net acid production may become considerably more complex than that described here. Neutral salts of metabolizable acids would cause removal of hydrogen ions from the body and thus would reduce net acid production without affecting the organic acid output in the urine. On the other hand, salts of noncombustible acid (e.g., benzoic or tartaric) would not affect acid production although they might well increase the total measured excretion of organic acids in the urine. Such considerations emphasize the importance of the synthetic diet in providing the conditions necessary for the accurate determination of net endogenous acid production.

In the present studies the presence of phosphate residues in the dietary protein appeared to be responsible for the third component of endogenous acid production. Previous opinion on the role of phosphorus in acid production has varied. Most of the earlier authorities (1, 2, 11–14) had assumed that *all* dietary phosphorus contributed to acid production. More recently, however, Hunt (5), Christensen (4, 15) and others have pointed out that, since most phosphorus in natural foods exists already oxidized as the potassium, magnesium, calcium or sodium salts of organic phosphoric acid monoesters, hydrolysis of such esters would result in the formation of essentially neutral inorganic phosphate with no liberation of protons.[3]

[3] Since organic phosphate monoesters in proteins probably have slightly lower pK'_2 values than inorganic phosphate (17) the cleavage of such neutral esters would probably have a slightly alkaline effect.

The metabolism of phosphodiesters might be expected, on the other hand, to yield phosphoric acid. However, in most diets diester linkages would occur relatively infrequently and would not often make a significant contribution to acid production. In support of such reasoning, Hunt reported that he was unable to observe any constant relationship between dietary phosphorus and urinary excretion of acid.

In the experiments reported here, the only source of dietary organic phosphorus was the purified soy phosphoprotein which was virtually free of inorganic electrolytes. In contrast to the situation prevailing in most natural foods, the phosphate residues in the soyprotein used in eight of the ten balances were neutralized by combustible cations in the form of arginine ($R\text{-}NH_3^+$) or ammonium. Such cations, when metabolized, ultimately must yield protons (see discussion in Methods). In calculating the acid contribution from this source, it was assumed that metabolism and hydrolysis of dietary protein was complete and that the liberated phosphate residues were titrated to a pH of 7.4. That such assumptions were at least approximately true is indicated by the reasonably close agreement of acid excretion with the total acid production calculated in this manner (Table II).

The observations made during the course of the four relatively prolonged balance studies merit some final comment. In the bicarbonate experiment, the prompt fall in renal acid excretion to very close to zero levels (Figure 1) provides strong support for the idea that Subject GdeF was in fact in acid-base equilibrium prior to the bicarbonate load. In the two phosphate experiments a similar conclusion may be drawn from the fact that relatively small increments in net acid excretion were promptly followed by rises in serum CO_2 content. The fact that adjustments in renal acid excretion following the bicarbonate or neutral phosphate load resulted in a final cumulative equality between calculated acid production and acid excretion in the urine is also of great interest, for it tends to indicate that administered or endogenous acid or alkali is not ordinarily excreted in significant quantities by any extrarenal route, such as the stool. A similar conclusion follows from the fact that essentially all of the extra endogenous acid derived from methionine could be recovered in the urine (6).

In all of the prolonged balance studies, the observed relationships between acid balance and the serum CO_2 content were entirely consistent with what would be expected from the interplay of two physiological variables such as acid production and excretion. Thus, whenever the net excretion of acid exceeded the calculated rate of production, serum CO_2 content tended to rise and, conversely, whenever production exceeded excretion, the CO_2 content fell.

In an earlier study of normal subjects given an acute acid load (18), it was demonstrated that calculation of acute changes in net acid balance was feasible, assuming a constant, though unknown, rate of endogenous acid production. The present results appear to offer a technique for measuring the actual net balance of protons and therefore may permit study of the absolute gain or loss of hydrogen ions under a variety of experimental and clinical situations. The method requires, however, that the subjects be in approximate nitrogen equilibrium, because catabolism of tissue protein would present difficulties in calculation of acid production similar to those already described with the use of natural dietary protein.

SUMMARY AND CONCLUSIONS

The purpose of this study was to determine whether it was possible to measure the total endogenous production of fixed acid and, by comparing this independently determined quantity with the net renal excretion of acid, to measure the net external acid balance of the body.

In a series of ten balance experiments, normal subjects in an apparently steady state were fed a liquid-formula diet which had as the nitrogen source a purified soy phosphoprotein essentially free of mineral cations. It was demonstrated that the net renal excretion of acid was closely matched by the sum of: *a*) inorganic sulfate in the urine, *b*) total "organic acids" in the urine, and *c*) the acid calculated to be released by the metabolism of the combustible cationic groups covering the phosphate residues in the dietary phosphoprotein. In three experiments net production or excretion of acid was temporarily altered and, at the end, when a steady state had been restored, the equivalence

of cumulated acid excretion with the cumulated production of acid could again be demonstrated.

These observations are taken to mean that, under the present conditions, the total net production of fixed acid arose from the oxidation of organic sulfur, plus the production of nonmetabolized organic acids from neutral precursors, and the release of protons during the metabolism of the organic cations neutralizing phosphate esters or during the hydrolysis of phosphate diesters in the dietary phosphoprotein, or both. However, when noncombustible cations neutralize the phosphate residues, no acid production can be attributed to the presence of phosphate monoesters in the protein.

The relationship between acid production measured in this way and the renal excretion of acid appears to be that of a true physiological equilibrium. Provided that body composition remains relatively constant during the period of study, it should be possible to apply this technique to the direct measurement of the external balance of acid under a variety of experimental and clinical conditions.

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HISTAMINE RELEASE AND ENDOTOXIN SHOCK IN THE PRIMATE*

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There is a growing interest in the pathogenesis and treatment of endotoxin shock as this type of shock becomes increasingly important in medical and surgical practices (1–4). Recent work has described the hemodynamic effect of endotoxin in the dog which is characterized by a sudden and severe hypotension following administration of a lethal dose of endotoxin (5, 6). Previous reports from this laboratory (7–10) have extended the earlier observations, and specific attempts have been made to gain an understanding of the basic mechanisms underlying the development of the irreversible phase of this type of shock (9–16). An extension of these studies has ascertained the degree of species variation in endotoxin shock (17, 18), and major differences have been noted between the hemodynamic responses of the dog and monkey.

Recent reports have suggested the prominent role of histamine in endotoxin shock in animals other than the primate (14, 19–23). The release of histamine in anaphylaxis has also been described (24, 25) with similarities noted between the vascular actions of histamine and endotoxin (8–10, 13, 26–31). A recent finding by Schayer (20, 21) has indicated an increase in histidine decarboxylase activity after endotoxin administration and in other forms of stress.

The purpose of the present investigation was to assess a variety of changes in the monkey, previously demonstrated in other species after endotoxin administration. Of primary interest are changes in blood histamine and histidine and the ultimate effects of their release in the primates. Results of this study confirm the view that histamine plays a crucial role in shock due to endotoxin and extend the suggestions of Schayer to include the basic mechanism of histamine release.

METHODS

Eight adult male and female monkeys of the Cynopithecoid group, *Cercocebus torquatus Atys* (Sooty Mangabey) were used in the present investigation. Animals weighed between 5.7 and 11.0 kg and were first anesthetized with ether followed by a suitable intravenous injection of sodium pentobarbital (Nembutal). The amount of Nembutal required to sustain anesthesia varied so that animals were maintained between planes 1 and 2 of the surgical stage by appropriate administration of the anesthetic. Blood samples from 6 monkeys were obtained from a catheter inserted into the femoral vein and advanced to the inferior vena cava. Estimations for blood histamine [1] and histidine [2] were carried out and the procedures for the analyses have been described (14, 19). The following blood determinations were made in 4 animals before and after endotoxin administration: platelet and white blood cell counts (32, 33), blood-urea-nitrogen (BUN) (34, 35), and blood creatinine (36). Mean systemic arterial blood pressures were recorded by means of a Statham strain gage, and registered on a Sanborn direct-writing recorder. Determinations of hematocrits and blood pH were carried out on most animals coincident with electrocardiograph and heart rate recordings in several experiments. In 2 animals the following parameters were determined: mean systemic arterial pressure, portal venous pressure, heart rate, hematocrit and blood pH. Lethal doses of *Escherichia coli* endotoxin (Difco), 5.2 to 13.0 mg per kg (average, 8.9), were intravenously administered after sampling of blood for control determinations. All animals were dead within 30 hours (average, 6 hours) after endotoxin injection with the exception of Monkey 7 which was sacrificed at approximately 13 hours. Gross autopsies were made immediately after death and a variety of tissue samples was taken for histological analysis.

RESULTS

Figure 1 illustrates changes in mean systemic arterial blood pressure, hematocrit, blood pH, histidine and histamine following injections of lethal doses of endotoxin in six monkeys. There was a variable effect on blood pressure, with all animals

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[1] 2-(4-Imidazolyl)-ethylamine.

[2] α-Amino-4 (or 5) -imidazolepropionic acid.

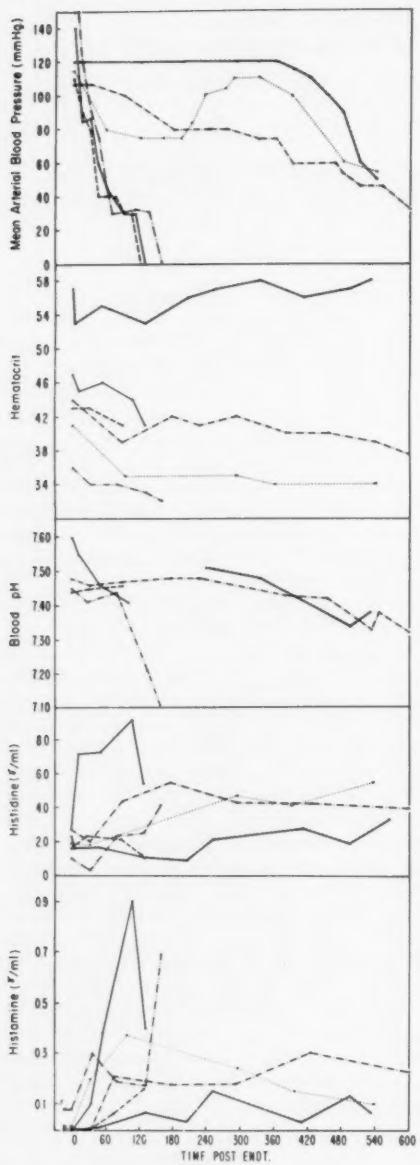

FIG. 1. CHANGES IN MEAN SYSTEMIC ARTERIAL BLOOD PRESSURE, HEMATOCRIT, BLOOD pH, HISTIDINE AND HISTAMINE AFTER INJECTIONS OF LETHAL DOSES OF ENDOTOXIN IN SIX MONKEYS. Abscissa: time in minutes post endotoxin. Ordinate: *animal* 1 ⊔⊔; 2 . . . . .; 3 .—.—; 4 ----; 5 —..—; 6 ———.

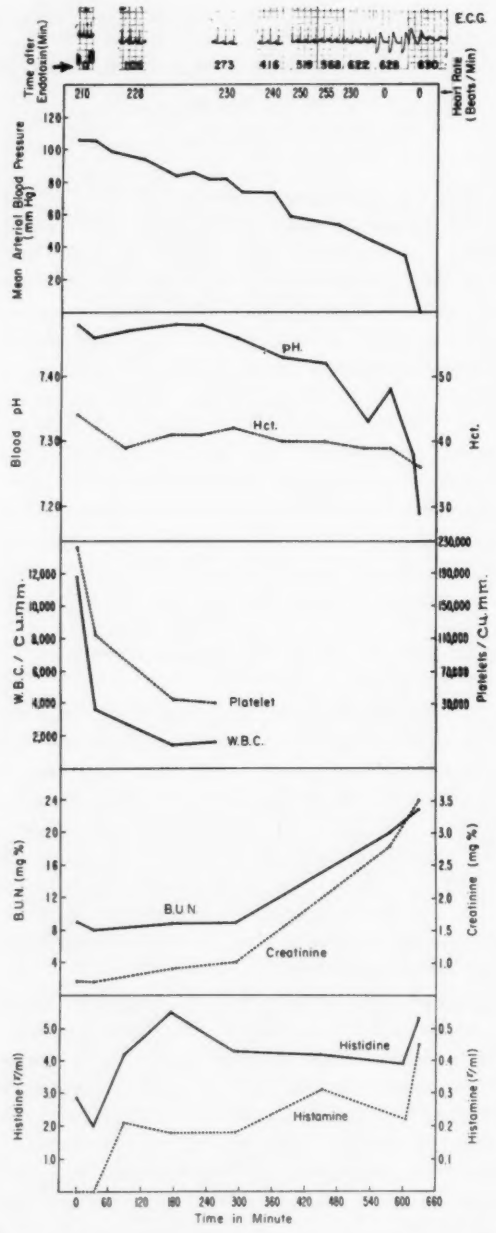

FIG. 2. RESULTS FROM A TYPICAL EXPERIMENT (MONKEY 3). Abscissa: time in minutes post endotoxin. Ordinate: note ECG tracing in upper frame. Pre-endotoxin paper speed, 10 mm/sec; postendotoxin paper speed, 25 mm/sec (time in minutes after endotoxin appears on each ECG section).

TABLE I

Platelet and white blood cell counts, blood-urea-nitrogen and blood creatinine determinations in four monkeys given lethal doses of endotoxin

Determination	Monkey no.	Control	Time post endotoxin in minutes 0–60	60–120	120–180	180–270	270–420	420–560
Platelets/mm³	3	224,000	114,000		36,000	32,000		
	4	196,000	56,000	96,000	*			
	5	88,000		34,000	17,000*			
	6	234,000	108,000	118,000	*			
White blood cells/mm³	3	11,800	3,550		1,400	1,600		
	4	14,600	1,800	2,300	*			
	5	27,500		10,000	4,350*			
	6	17,750	4,700	4,050	*			
BUN (mg%)	3	9	8		9	9	20	23
	4	11	13	16	17*			
	5	8		10	11–12*			
	6	14	15	17	*			
Blood creatinine (mg%)	3	0.7	0.7		0.9	1.0	2.8	3.5
	4	1.6	1.8	2.7	2.8*			
	5	0.9		1.3	1.5*			
	6	1.2	1.5	2.4	*			

* Death occurred during this time interval.

eventually becoming hypotensive within 8 hours after endotoxin. Hematocrits showed a steady decline except in Experiment 1 in which an early fall was followed by a return to the control value. The progressive development of acidosis was evident in most animals after endotoxin. Changes in blood histidine were variable, in most instances showing a significant rise above control values. All experiments demonstrated large increases of blood histamine, which became evident within the first hour after endotoxin injection.

Table I gives the values for platelet and white blood cell counts, BUN and creatinine for four of the six animals reported in Figure 1. There are marked decreases in platelet and white cell counts in all monkeys, although the time after endotoxin injection during which the lowest values were observed was variable. Significant increases in BUN and creatinine were observed in all animals within 1 to 2 hours after endotoxin administration.

Figure 2 designates the changes in a variety of parameters in Monkey 3. Steady decreases in

TABLE II

Blood pressures, heart rates, hematocrits and pH determinations in two monkeys given lethal doses of endotoxin

Measurement	Monkey no.	Control	Time post endotoxin in minutes 0–15	15–30	30–120	120–240	250–480	480–780
Mean systemic arterial blood pressure (mm Hg)	7	115	100	105	75	87	124	117
	8	110	100	90	37	30	18*	
Portal venous pressure† (mm Hg)	7	7.0	7.5	7.2	7.6	8.3	9.1	9.8
	8	7.0	8.0	7.0	7.0	6.5	7.7*	
Heart rate (beats/min)	7	185	190	190	207	202	204	220
	8	200	195	200	188	187	158*	
Hematocrit	7	47.5	48.0		47.0	48.0	52.5	54
	8	38.5	35.5		37.0	37.0	37.0*	
Blood pH	7		7.41		7.40	7.44	7.40	7.35
	8	7.43	7.43		7.42	7.36	7.14*	

* Death occurred during this time interval.
† Portal venous pressures indicate maximal readings for the time indicated.

TABLE III

Histological findings in five monkeys given lethal doses of endotoxin

Organ	Monkey no. and sex				
	1 ♂	2 ♀	3 ♀	4 ♀	5 ♀
Adrenal	Marked congestion	N	N	N	
G.I. tract	N*	N	N	N	N
Heart	Small vessels engorged; one small area of hemorrhage	N	N	N	N
Kidney	Marked capillary and glomerular engorgement; in some tubules cells swollen, cytoplasm fluffy	N	N	Early tubular epithelial degeneration predominantly in cortical region	
Liver	Congested; no cellular alterations	N	N	N	Multiple foci round cell infiltrate, old
Lung	Patchy cellular infiltrate	N	N	N	N
Pancreas		N	N	N	N
Spleen	Congested	N	N	N	N
Time of death post endotoxin (min)	840	1,600 (est.)	630	120	160
Dose of endotoxin (mg/kg)	13.0	5.2	10.4	10.1	10.0

* Normal.

mean arterial pressure, blood pH and hematocrit are observed after injection of endotoxin. Sudden decreases in platelet and white cell counts are noted, while the blood levels of creatinine, BUN, histamine and histidine show marked increases during the 10 hour postendotoxin period. The electrocardiograph was normal until 622 minutes after endotoxin, just prior to death.

Table II lists a number of changes in two monkeys after administration of endotoxin. Animal 7 did not become hypotensive during the entire period of observation and was subsequently sacrificed. The second monkey exhibited a progressive decrease in arterial blood pressure with death occurring at approximately 7 hours. Portal venous pressures changed very little during the postendotoxin period. A progressive decrease in heart rate of Animal 8 was evident as hypotension developed. A slight degree of hemoconcentration was seen in Monkey 7, although changes in hematocrit in the second animal were negligible. A significant decrease in pH occurred in Monkey 8.

All of the electrocardiograms were normal until just prior to death when the systemic blood pressure was extremely low.

The results of the histological sections are shown in Table III. The differences seen in the sections of Monkey 1 may be associated with respiratory difficulty and cyanosis, noted after induction of anesthesia, which were corrected by intubation and aspiration of pulmonary secretions. No other consistent histologic changes were noted except those observed in the kidneys of two animals.

DISCUSSION

Role of histamine in endotoxin shock. The large increases of blood histamine reported in the present study may account for the progressive development of systemic hypotension observed in the monkey given a lethal dose of endotoxin. An increased blood level of histamine could act on the peripheral vasculature to effect a decrease in the total peripheral resistance. Vascular resistance is known to decrease in various vascular beds of the dog (9, 10) after the injection of endotoxin. The present findings give further support for the role of histamine as a "shock toxin" (20, 21).

The mechanism of histamine release in endotoxin shock. It has recently been proposed by

Schayer (20, 21) that the rate of histamine synthesis is accelerated in endotoxin shock because of the increased activity of histidine decarboxylase. The findings of the present study extend his view to account for the increase in histamine on the basis of a rise in histidine, which supports the concept that endotoxin induces new histamine synthesis. Previous work in dogs (19) has indicated that the progressive increase of the histamine: histidine ratio is related to the relative rates of formation, conversion and destruction of the two components in question. It is possible that a rise in plasma histamine has resulted because of the marked decrease in the numbers of circulating platelets and white blood cells (37, 38). Similar decreases have been reported in the dog administered endotoxin (22) and in various forms of shock in man and animals (39–41). Histamine may possibly be released from the bound form in whole blood to the circulating form in plasma (25, 42), and from tissue such as muscle (43) or lung (44), or from various other sources (37).

Species variations in endotoxin shock. Major differences have been noted between the hemodynamic responses (1, 17, 18) and histological findings (1) of the dog and monkey after injections of lethal doses of endotoxin. The gradual fall in systemic arterial pressure, the small increase in portal venous pressure and the postmortem tissue findings in the present series of monkeys were similar to those in monkeys of a different species (1, 18). These findings are in marked contrast to those observed in dogs (5, 6, 45). The relative phylogenetic proximity of monkey to man gives added interest to the observations of gross species differences. The common hemolysis and hemoconcentration in dogs given lethal doses of endotoxin (18, 26–28) were not apparent in the monkeys of the present study. No cardiac abnormality was observed in the monkeys' electrocardiographic tracings until just prior to death, this observation being similar to that of dogs (6).

Correlation with human bacteremic shock. Case studies of bacteremic shock in man have been reported (2–4). Similarities in the findings of the monkeys of the present study and those in man are evident. Kidney disturbances have been reported in man after infections due to gram-negative bacilli (2, 3). Renal dysfunctions have included anuria, oliguria, hematuria and uremia which are usually conceded to be precipitated by the effects of systemic hypotension and consequent renal ischemia (4). Findings suggestive of ischemic changes in the kidneys were noted in the present study, and this observation is consistent with changes seen in human bacteremic shock (1–4, 46–48). The finding of an elevated blood creatinine and BUN in man and monkey suggests that renal failure is common to both. Other similarities noted were changes in hematocrit and blood pH. Further basic and clinical investigations are needed to provide a more thorough understanding of the intimate mechanism of bacteremic shock.

SUMMARY

The increasing interest in the pathogenesis and treatment of endotoxin shock has prompted the present investigation. Eight monkeys were given lethal doses of endotoxin and a number of parameters studied. Results indicate the prominent role of histamine in endotoxin shock and extend the suggestions of Schayer to include the basic mechanism of histamine release. Species variations were noted which stress the importance of the close phylogenetic proximity of monkey to man. Relationships between human and experimental bacteremic shock have been indicated.

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CORRECTION

On page 1133 of the article entitled "Inhibition of L-Thyroxine Monodeiodination by Thyroxine Analogs" by Frank C. Larson and Edwin C. Albright (J. clin. Invest. 1961, **40**, 1132), three molar concentrations were omitted from Table I. The subheadings for columns 1, 2 and 3 of the table under the heading, "Per cent inhibition with increasing molar concentrations," should have been "10^{-7}, 3×10^{-7} and 10^{-6}," respectively.

FLUORESCENT-STAINABLE ANTIBODIES TO THE EATON AGENT IN HUMAN PRIMARY ATYPICAL PNEUMONIA TRANSMISSION STUDIES *

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The etiology of those cases of nonbacterial pneumonia that cannot be associated with the viruses of psittacosis and influenza, adenoviruses, or other well delineated agents, and in which cold hemagglutinins or streptococcus MG agglutinins often develop, has been a controversial subject. The possible role in this disease of an agent isolated by Eaton, Meiklejohn, van Herick and Talbot in 1941 from patients with primary atypical pneumonia (1) has been difficult to evaluate because of the agent's limited host range and technical problems in performing specific serologic tests with it. Eaton's agent can be propagated in embryonated hens' eggs, as demonstrated by production of pneumonia on passage to cotton rats (*2*), but produces no apparent pathologic change in the chick embryo (2, 3). Using a modification of the fluorescent antibody technique of Coons and Kaplan (4), Liu, Eaton and Heyl have provided both a means of localizing the antigens in the chick embryo and a method by which sera can be examined for fluorescent-stainable antibodies to Eaton's agent (3, 5). Additional isolations of Eaton's agent from patients and demonstrations of fluorescent-stainable antibodies to it have been reported by Cook (6) and Chanock (7) and their associates, confirming and extending the observations of Eaton and Liu. The specificity of this fluorescent antibody reaction in terms of the etiology of the disease has been questioned (8), however, since patients with primary atypical pneumonia may develop a number of nonspecific serologic responses (9–15). Primary atypical pneumonia produced under experimental conditions in man would provide an ideal situation for comparison of these various serologic tests.

In 1944 the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board performed studies on the transmission of primary atypical pneumonia to human subjects, using sputa and throat washings from patients with the characteristic syndrome (16a–d). Among the volunteers inoculated, two distinct disease patterns were induced: *1*) an illness with constitutional symptoms and physical and roentgenographic findings of primary atypical pneumonia, and *2*) a minor respiratory illness with minimal constitutional symptoms and physical findings but without pulmonary infiltration. Two similar experiments were performed, those men developing primary atypical pneumonia in the first experiment serving as donors of inocula for the second. Most of the cases of primary atypical pneumonia and a few cases of the milder respiratory illness were associated with rises of cold hemagglutinins. The study suggested the presence of a filtrable, transmissible agent associated with the induced disease, but none was isolated by methods then available.

Further information on the association of Eaton's agent with human disease has been afforded by application of Liu's techniques to preserved sera from participants in the above transmission studies. Significant rises of fluorescent-stainable antibodies to Eaton's agent occurred in the majority of inoculated volunteers' sera. The data obtained are presented in this report.

MATERIALS AND METHODS

Eaton's agent. The Mac strain of Eaton's agent (17) was used throughout the experiments, and seed material was kindly supplied in the form of infected chick embryo

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TABLE I

Summary of clinical and serological data, first primary atypical pneumonia volunteer experiment

Inoculum	Case no.*	Disease induced†	Incubation period‡	Severity of illness	Serological data‖			
					Cold hemagglut. (max. titers)§	Fluorescent-stainable antibody		
						Pre-inoc.	Postinoculation	
							Titer	Day bled
			days					
Untreated								
	4¶	PAP	8	Severe	512**	10	320	32
	9	MRI	4	Mild	<8	N.A.	N.A.	
	11	MRI	4	Mild	<8	<10	80	15
	13	MRI	6	Mild	16	<10	40	25
	15	MRI	3	Mild	<8	<10	20	18
	28	MRI	1	Mild	8	320	320	25
	30	MRI	2	Mild	<8	10	20	28
	31	MRI	6	Mild	<8	<10	<10	25
	32	MRI	1	Mild	<8	<10	160	25
	34	MRI	1	Mild	8	10	20	28
	42¶	PAP	8	Moderate	32	N.A.	N.A.	
	44¶	PAP	8	Moderate	2,048	<10††	<10††	18
Filtered								
	2¶	PAP	8	Moderate	16	N.A.	N.A.	
	7	0			<8	<10	80	25
	14	MRI	4		8	10	10	25
	17	0			<8	<10	<10	28
	20	0			<8	<10	40	18
	24¶	PAP	9	Severe	512	<10	<10	32
	25	PAP	12	Moderate	<8	<10	<10	28
	27	MRI	10	Mild	<8	<10	80	18
	29	MRI	11	Mild	<8	N.A.	N.A.	
	33	MRI	9	Mild	<8	<10	<10	25
	35	MRI	7	Mild	8	<10	20	25
	45¶	PAP	12	Mod. severe	256**	<10	320	32
Autoclaved								
	1	0			<8	20	10	28
	3	0			<8	<10	20	32
	5	PAP	8	Mod. severe	16	<10	40	32
	6	0			<8	<10	<10	28
	10	0			<8	<10	<10	25
	16	0			<8	<10	<10	25
	18	0			<8	<10	40	25
	19	0			<8	N.A.	N.A.	
	22	MRI	7	Mild	<8	< 8	320	18
	26	0			<8	<10	10	28
	46	PAP	10	Moderate	<8	<10	10	18
	47	PAP	10	Moderate	32	10††	160††	32

* Case numbers refer to those from the original volunteer experiments (Ref. 16a-d).
† Diseases: PAP = primary atypical pneunonia; MRI = minor respiratory illness; 0 = no illness (see text).
‡ Time between inoculation and first symptoms.
§ Maximal cold hemagglutinin titer obtained in weekly tests after inoculation; pre-inoculation titers all <8 unless otherwise specified.
‖ N.A. = sera not available.
¶ Donors of inocula for the second volunteer experiment.
** All these cases developed streptococcus MG agglutinin titers of 32 (from <8).
†† Titrations performed by Dr. C. Liu.

lung suspension by Dr. Chien Liu. The agent was propagated by amniotic stab inoculation of 14-day old chick embryos with 0.2 ml each of a 1:5 dilution of seed material in brain-heart infusion broth (Difco) and 1 per cent bovine albumin (Armour). For the first two passages 1,000 U of penicillin was added per ml of lung suspension. After 5 days' incubation at 35° C, inoculated embryos were harvested without chilling, and the lungs and trachea of each were removed aseptically. Tissues were ground in 2 ml of the broth-bovine albumin mixture per specimen in a glass tissue grinder. The pooled crude suspension was divided into 1- to 2-ml aliquots, sealed in glass ampules, and stored at −70° C. Material used in the experiments to follow was from the sixth egg passage, having an infectivity titer of 10^6 EID_{50} per 0.2 ml, as determined by methods described by Liu (3).

Eggs. White Leghorn eggs from a local source were used exclusively. These were placed in a commercial-type incubator-hatcher for 14 days and after inoculation were maintained in a humidified standard laboratory incubator.

Sera. Sera collected before and after inoculation (see Tables I and II, and References 16a–d) of the volunteers were stored at 4° C for 12 to 18 months before lyophilization. They were then kept at room temperature for about 8 years and were subsequently stored at 4° C. Sera from 70 of the original 78 participants were available for examination. Each specimen was reconstituted to the original volume by addition of 2 ml of sterile distilled water, and aliquots were removed for testing. Sera from patients with primary atypical pneumonia in the Cleveland area (18), stored at −20° C since 1947, provided positive and negative control specimens. Results of previous titrations of these control sera have been published by Cook and co-workers (6).

For the preparation of fluorescein isothiocyanate-con-

TABLE II

Summary of clinical and serological data, second primary atypical pneumonia volunteer experiment *

Inoculum	Case no.	Disease induced	Incubation period	Severity of illness	Serological data: Cold hemagglut. (max. titers)	Serological data: Fluorescent-stainable antibody: Pre-inoc.	Serological data: Fluorescent-stainable antibody: Postinoculation: Titer	Serological data: Fluorescent-stainable antibody: Postinoculation: Day bled
			days					
Untreated								
	101	0			<8	<10	160	32
	103	MRI	6	Mild	128	<10	160	21
	111	0			<8	10	160	29
	112	PAP	7	Mod. severe	512	<10††	<10††	42
	126	0			<8	10	20	42
	127	MRI	6	Mild	32	N.A.	N.A.	
	129	MRI	7	Mild	16**	<10	640	29
	131	MRI	4	Moderate	256	N.A.	N.A.	
	132	0			16	10	40	46
	137	PAP	8	Severe	8,192	<10	320	73
	138	MRI	7	Moderate	16	<10	80	21
	158	PAP	7	Moderate	<8	<10	80	29
Filtered								
	105	PAP	14	Moderate	1,024	<10	160	21
	110	MRI	15	Mild	64 (pre-inoc. 8)	80	320	42
	113	MRI	10	Mild	<8	<10	40	29
	120	0			<8	<10	40	29
	124	MRI	9	Moderate	8	<10	80	46
	130	MRI	12	Mild	16	<10	80	43
	134	MRI	12	Moderate	64	<10	20	65
	136	0			<8	<10	<10	29
	142	0			<8	<10	40	21
	144	PAP	13	Moderate	128	<10††	80††	29
	146	PAP	12	Moderate	1,024	<10	20	50
Autoclaved								
	102	MRI	19	Mild	32	N.A.	N.A.	
	106	0			<8	20	20	41
	107	0			<8	20	20	42
	108	0			<8	<10	10	20
	109	0			<8	<10	<10	27
	115	0			<8	20	10	46
	116	0			<8	<10	<10	27
	117	0			> 32 (pre-inoc. 8)	20	80	29
	118	0			<8	10	10	43
	119	0			<8	40	40	29
	120	0			<8	<10	40	29
	121	0			<8	<10	<10	29
	125	0			<8	<10	20	43
	133	0			<8	<10	20	43
	135	0			<8	<10	<10	42
	139	0			<8	<10	10	21
	140	0			8	10	20	43
	141	0			<8	<10	<10	43
	143	0			<8	<10	20	43

* See footnotes to Table I.

jugated antisera, adult goats were immunized by weekly subcutaneous injections of pooled human globulins with aluminum hydroxide as adjuvant. Injections were continued until the undiluted serum produced a precipitin reaction with antigen dilutions of 10^{-4} or greater.

Fluorescein-conjugated antihuman globulin solution. The globulin fraction of the goat antihuman serum was precipitated with $(NH_4)_2SO_4$ by standard methods (19). The globulin solution was conjugated with fluorescein isothiocyanate (20)[1] by the technique of Marshall, Eveland and Smith (21). The final product contained 1 per cent protein and 0.05 mg fluorescein isothiocyanate per mg of protein. After dialysis against phosphate-buffered saline (0.01 M, pH 7.2) for 7 to 8 days to remove uncombined fluorescein, the labeled globulin solution was absorbed once each with acetone-extracted chick embryo and adult chicken lung powders (4) to remove nonspecific staining properties. The absorbed, conjugated globulin solution was stored at −25° C until needed.

Fluorescent microscopic equipment. The source of ultraviolet light was an AH-6 water-cooled mercury vapor lamp,[2] filtered by a 2.5 cm path of 2.5 per cent $CuSO_4 \cdot 5\ H_2O$ solution and a half-thickness Corning 5840 filter. A standard laboratory monocular microscope was employed, fitted with a cardioid dark field condenser and a double Wratten 2-A gelatin filter in the ocular.

Preparation of infected tissue for serum titrations. Chick embryos were inoculated with stock virus suspension and harvested as described above. The thorax of each embryo was removed, placed in an individual rubber-stoppered tube and quick-frozen in an ethanol-carbon dioxide ice bath. In a microtome cryostat[3] 4-μ frozen sections of the lungs of each embryo were cut. These were examined for viral antigens by the indirect fluorescent antibody technique, with known positive human convalescent serum and goat antihuman fluorescein-conjugated globulin solution. Embryos showing maximal (4+) fluorescence of the bronchial epithelium in two or more foci were selected for future use. Series of such sections were prepared for titrations, dried at room temperature, and stored at 4° C until used (within 1 week). Several hundred sections from each specimen could be obtained. If screening revealed antigen in the first and last sections of a consecutive series, those between were assumed to be positive.

Serum titrations. Fluorescent-stainable antibodies were titrated by a modification of the technique of Weller and Coons (22) as described by Liu and co-workers (5). Simultaneous titrations were performed, using sections from the same chick embryo, on pre- and postinoculation sera of a given individual. Sera were first inactivated by heating at 56° C for 30 minutes. Dilutions were prepared in an ice bath; the diluent was 1:20 fresh human serum, free from stainable antibodies to the Eaton agent, in phosphate-buffered saline. Sera negative at a screening dilution of 1:10 were not further evaluated, while those positive were diluted in twofold increments to 1:1,280 for testing on the same day. Each serum dilution tube received a code number and was not further identified until completion of the titration experiments. In addition, all titrations were performed without knowledge by the observer of the volunteer's classifications.

Indirect fluorescent antibody technique. The entire staining procedure was conducted at room temperature. Series of infected chick embryo lung sections were fixed in acetone for 10 minutes and dried at 36° C for 20 minutes. Each section was overlaid with one serum dilution for 30 minutes. Slides were washed with gentle agitation in buffered saline for 10 minutes, wiped dry except for the section, and a drop of goat antihuman fluorescein-conjugated globulin solution applied for 30 minutes. The slides were again washed in buffered saline and mounted in buffered glycerol. The sections were examined under a fluorescence microscope and the amount of fluorescence recorded as 1 to 4+. Titration endpoints were determined as those serum dilutions giving minimal (1+) fluorescence of the chick bronchial epithelium.

Titration and staining controls. Dilutions of 1:10, 1:100, and 1:1,000 of a serum with a consistent titer of 1,280 were included in each series of titrations to indicate the range and reproducibility of positive reactions. In addition to the positive serum, the following controls were included in each series of titrations with identical processing: *1*) the first and last sections from each consecutive series used, stained with a 1:10 dilution of positive control serum; *2*) sections stained with the serum diluent (1:20 fresh known-negative human serum in buffered saline); and *3*) sections held in buffered saline during the serum-staining period.

RESULTS

Detailed reports of the original experiments, which were designed to evaluate the nature of the etiological factors and clinical characteristics of primary atypical pneumonia, have been published previously (16a–d). A brief summary of these experiments is included here, however, for its pertinence to the material to follow.

First experiment (*Table 1*). Pooled sputa and throat washings from 6 patients with primary atypical pneumonia were divided into 3 aliquots which were respectively autoclaved, filtered to remove bacteria, and retained untreated. Three groups of volunteers, totaling 37 individuals, each received one of the types of inocula by nasopharyngeal insufflation, those inoculated with autoclaved material serving as the control group. Ten cases of primary atypical pneumonia and 15 cases of an undifferentiated minor respiratory illness resulted. These findings were difficult to interpret because both disease syndromes occurred in the control group. A modified repetition of this study was performed for further evaluation.

[1] Dajac Labs., Borden Chemical Co., Philadelphia, Pa.
[2] General Electric Co., Cleveland, Ohio.
[3] Harris Refrigeration Co., Cambridge, Mass.

Second experiment (Table II). Inoculum was obtained from 6 of the more severe cases of primary atypical pneumonia which had been induced in the first experiment, and aliquots were again autoclaved, filtered, and retained untreated. In addition to more rigorous isolation procedures, the inoculations were performed outdoors and on different days, beginning with the autoclaved material and concluding with the untreated pool. Six cases of primary atypical pneumonia and 10 cases of minor respiratory illness developed in the 24 men who received untreated or filtered inocula. No clinical illnesses developed in the control group except in one volunteer (Case 102) who violated isolation restrictions by visiting a patient with minor respiratory illness (Case 138).

The original serological data from the two experiments are summarized in Tables I and II. Fourfold or greater responses of cold hemagglutinins occurred in the donors of inocula, in most of the volunteers developing primary atypical pneumonia, and in a few of those with minor respiratory illness. Streptococcus MG agglutinin responses occurred rarely.

The introduction of a new serological procedure for the study of primary atypical pneumonia made it possible to re-evaluate these data, and in so doing to expand information concerning the nature of the test itself. The significance of the results of fluorescent-stainable antibody titrations on the sera from the volunteers was judged by: *1*) correlation with the types of inocula administered; *2*) correlation with the occurrence of induced illness; and *3*) comparison with the previously reported results of cold hemagglutinin and streptococcus MG agglutinin tests performed on the same sera.

The data obtained revealed an association between illness and the development of fluorescent-stainable antibody to Eaton's agent. In addition, a number of volunteers who remained well after inoculation and failed to show cold hemagglutinins or streptococcus MG agglutinins developed these antibodies. Certain discrepancies concerning the development of illness and antibody responses will be detailed and discussed.

Relationship of fluorescent-stainable antibody responses to types of inocula. The correlation between the nature of the inoculating material and the fluorescent-stainable antibody responses provided one method of assessing the significance of the results obtained. The outcome of inoculation with filtered or untreated material was similar in both experiments; therefore, these groups have been combined for presentation, as indicated in Table III. Sixty-eight per cent of those receiving filtered inocula and 65 per cent of those receiving untreated inocula developed fourfold or greater rises of fluorescent-stainable antibody. The mean antibody response of the former group was a 10-fold increase, while that of the latter was a 21.9-fold increase. It was previously noted that those men receiving filtered inocula had somewhat milder illnesses after longer incubation periods, which was thought to be due to a reduction of the inoculating dose occasioned by the filtration procedure (16c) (Tables I and II).

In the first transmission experiment, three cases of primary atypical pneumonia and one of minor respiratory illness developed in the group receiving autoclaved inoculum. These unexpected illnesses were clinically and serologically identical with those induced in volunteers given active (filtered and untreated) inocula (Tables I and II). In view of the absence of apparent disease in the control group of the second experiment, it was felt that the illnesses in the first instance were produced by inadvertent infection rather than by stimulation of a theoretical latent agent by the inoculation procedure (16b). Possible mechanisms involved have been previously discussed (16b), and included contamination of the air-pump used for insufflation of inocula, airborne spread at the time of inoculation, or cross-infection after inoculation. The second possibility was favored by the occurrence of primary atypical pneumonia in an attendant and two members of the professional staff involved.

Since inadvertent infection apparently occurred in the first control group, this has been excluded from the comparative data to be presented. Of the 17 control subjects with available sera from the second experiment, 3 developed 2-fold anti-

TABLE III

Relationship of fluorescent-stainable antibody responses to types of inocula received by 59 volunteers

Type of inoculum	Cases	4-Fold or >rise		Mean rise of titer*
	no.	*no.*	*%*	*fold*
Autoclaved†	17	4	23.5	1.3
Filtered‡	22	15	68.2	10.0
Untreated‡	20	13	65.0	21.9

* Average-fold change based on the responses of all individuals in each group.

† Control group of second experiment.

‡ Combined groups from first and second experiments.

body rises, and 4 showed 4-fold rises, giving a mean response for the group of 1.3-fold (Table III). In one of the latter instances (Case 117, Table II), the cold hemagglutinin titer began to rise 2 weeks prior to inoculation, suggesting an unassociated infection. Using the rank-sum method (23), the differences in mean responses of the groups receiving either filtered or untreated inocula, compared with the control group of the second experiment, were found to be statistically significant ($p < 0.01$). The difference between the groups receiving filtered or untreated inocula was not significant.

Relationship of fluorescent-stainable antibody responses to the volunteers' postinoculation diagnoses. The manifestations of the two types of induced illnesses have been detailed previously (16c) and are outlined briefly above. The cases of primary atypical pneumonia followed an incubation period of 7 to 14 days, while the undifferentiated minor respiratory illneses had a more variable incubation period of 1 to 19 days. The possibility that these two clinical entities may have been caused by different etiologic agents was neither confirmed nor disproved by the original data (16b). Results from the present study made possible further evaluation of this problem.

Fourfold or greater rises of fluorescent-stainable antibody to Eaton's agent developed in the sera of 21 of 31 individuals who became clinically ill after receiving active inocula (Table IV). These included 7 of 11 cases of primary atypical pneumonia and 14 of 20 cases of minor respiratory illness occurring in the two transmission experiments. Seven of 11 men who remained well after inoculation also developed significant rises. All of the patients with primary atypical pneumonia had pre-inoculation antibody levels of less than 10, while among those developing minor respiratory illness, two had baseline titers of 10, one of 80, and one of 320. In the group remaining well, three volunteers had pre-inoculation titers of 10 and one of 20 (Tables I and II).

TABLE IV

Relationship of fluorescent-stainable antibody responses to diagnosis in 42 volunteers given active inocula

Diagnosis*	Cases	4-Fold or >rise		Mean rise of titer
	no.	*no.*	*%*	*fold*
Primary atypical pneumonia	11	7	63.6	24.0
Minor respiratory illness	20	14	70.0	15.4
No illness	11	7	63.6	8.5

* Clinical diagnosis by criteria listed in the text.

No essential differences in the percentage of significant responses occurred among the ill and the well groups (Table IV). Calculation of mean increases of titer revealed that the response of the primary atypical pneumonia group was 24-fold; that of the minor respiratory illness group, 15.4-fold; and that of the group without illness, 8.5-fold. While these figures represent a progressive trend, the differences between groups are not statistically significant when tested with the rank-sum method ($p > 0.05$).

Relationship of fluorescent-stainable antibody responses to cold hemagglutinins and streptococcus MG agglutinins. It has been shown that cold hemagglutinin responses in primary atypical pneumonia bear a direct relationship to the severity and

TABLE V

Relationship of antibody responses to the severity of disease among 42 subjects receiving active inoculum

Degree of illness*	Cases	Fluorescent-stainable antibody			Cold hemagglutinins		
		4-Fold or >rise		Mean rise	4-Fold or >rise		Mean rise
	no.	*no.*	*%*	*fold*	*no.*	*%*	*fold*
Not ill†	11	7	63.6	8.5	0	0.0	0.0
Mild‡	17	11	64.7	16.0	5	29.4	3.9
Moderate§	8	6	75.0	12.5	5	62.5	102.8
Severe‖	6	4	66.7	32.7	6	100.0	458.7

* Based upon clinical appraisal, extent of pulmonary infiltration, and duration of illness.
† Patients without clinical disease.
‡ Patients with an undifferentiated minor respiratory illness.
§ Patients with febrile respiratory disease and rales without pulmonary infiltration demonstrable radiographically, and cases of mild primary atypical pneumonia.
‖ Patients with moderately severe and severe primary atypical pneumonia.

TABLE VI

Correlation between responses of both fluorescent-stainable antibody and cold hemagglutinins in the sera of 42 volunteers

Cold hemagglut.	Fluorescent-stainable antibody	
	Present*	Absent†
Present*	15	3
Absent†	13	11

* Fourfold or > titer rise.
† Fourfold titer rise.

duration of illness (9, 13). That this was the case in the studies under consideration is illustrated in Table V. Volunteers with more severe illnesses demonstrated both a higher level and a greater percentage of cold hemagglutinin rises. The development of fluorescent-stainable antibody did not follow this pattern in occurrence, roughly two-thirds of volunteers in all clinical categories having fourfold or greater antibody responses. There was a trend toward higher titers in those men with the more severe illnesses, as shown by comparison of mean antibody increases for the same groups, but these differences were not statistically significant.

The correlation between the development of cold hemagglutinins and fluorescent-stainable antibodies in the same sera is illustrated in Table VI. Fourfold or greater cold hemagglutinin responses occurred in 18 (43 per cent) of 42 individuals receiving active inocula. Of these 18 patients having significant cold hemagglutinin responses, 15 (82 per cent) also had rises of fluorescent-stainable antibody; however, of the 28 with significant fluorescent-stainable antibody rises, only 15 (54 per cent) had cold hemagglutinin responses. Rises of streptococcus MG agglutinins, all sixfold, occurred in two patients with primary atypical pneumonia and in one with minor respiratory illness. Significant responses of both cold hemagglutinins and fluorescent-stainable antibody developed in these same patients. No detectable antibody responses occurred in 11 of the 42 volunteers (26 per cent), including 4 without illness, 6 with minor respiratory illness, and 1 with primary atypical pneumonia.

DISCUSSION

Although the identity of the infectious agent involved in the experiments is unknown, the rises in titer of fluorescent-stainable antibody to the Eaton agent were striking. Interpretation of the data is complicated by the present lack of knowledge of the nature of these fluorescent-stainable antibodies. Patients with primary atypical pneumonia may develop a variety of abnormal serologic reactions during convalescence: agglutinins for group O erythrocytes in the cold; agglutinins for streptococcus MG; complement-fixing reactions for various heterologous tissue antigens; and even false serologic tests for syphilis (8–15). Liu and associates, by means of cross-absorption experiments, demonstrated that the fluorescent-stainable antibodies are distinct from cold hemagglutinins and streptococcus MG agglutinins (5). The antigen-fluorescent-stainable antibody reaction does not bind complement but is enhanced by the presence of a heat-labile factor in normal fresh serum (5, 24). Except in rare instances (25), sera containing these antibodies fail to neutralize Eaton's agent in eggs; but Eaton has shown that sera of patients with primary atypical pneumonia may neutralize the agent, as indicated by prevention of pneumonia in cotton rats or hamsters inoculated with serum-virus mixtures (26, 27). Results of both fluorescent-stainable antibody titrations and cotton rat neutralization titrations on the same sera reveal a close correlation in development of the two (5, 28). The data presented above suggest that the presence of fluorescent-stainable antibodies did not provide complete protection from induction of disease in man, although modification of disease may have occurred in those individuals who had pre-existing antibody at a measurable level. The data from this series are quite limited, but none of the individuals receiving active inocula who developed primary atypical pneumonia had a baseline titer of 10 or greater, while several of those developing minor respiratory illness or remaining well had baseline titers above this range.

Certain discrepancies occurred in the experimental data which are difficult to interpret. Although the control group of the second experiment appeared adequate, fluorescent-stainable antibodies in titers considered to be significant developed in a few cases. Possible reasons for these responses are: *1*) that there were undetected instances of cross infection in addition to the one known (Case 102, Table II); *2*) that the denatured antigen in

the autoclaved inoculum was sufficient to evoke an immune response; and *3*) that the sensitivity of the fluorescent-stainable antibody test as performed was too great. No data are available to test the first two hypotheses; limited experimental data have been accumulated to test the third possibility. By determining the fluorescent-stainable antibody titers simultaneously in multiple aliquots of the same serum, it was found that a variation by chance of 2 tubes (fourfold) would occur less than once in 20 times (28). This suggests that the responses seen in the control group are significant. The mechanism of these responses remains unexplained.

Not all of the patients with primary atypical pneumonia developed fluorescent-stainable antibody responses, including some considered to have had severe disease. It is possible that the negative reactions could be explained by loss of antibody through deterioration of the specimens. Another possible explanation for the negative reactions was that more than one agent was transmitted in the volunteer studies, especially since the inocula were obtained by pooling material from several patients. Sera tested from four of the six donors for the second experiment revealed significant fluorescent-stainable antibody responses to Eaton's agent in two instances (Table I). Sera from the donors for the first experiment were not available for testing. Repeated attempts to isolate the responsible agent(s) from lyophilized samples of the original inocula, using eggs and a variety of tissue culture cell lines, have all been unsuccessful (28). Additionally, failure to demonstrate antibody in some patients' postinoculation sera may be related to the time they were collected. Some of the samples were obtained 2 to 3 weeks after inoculation of the volunteers, or as early as 7 days after the onset of illness (Tables I and II), and Liu has demonstrated that the maximal percentage positivity of sera for fluorescent-stainable antibody may not occur until the fourth week of illness (5).

Some of the deficiencies in this series may be negated by appraisal of the results in terms of those that might be anticipated in the transmission of respiratory disease to human subjects. Table VII summarizes the results of several studies from the literature in which agents of known pathogenicity were employed. The over-all illness rate of 73.8 per cent in the primary atypical pneumonia experiments compares favorably with those of transmission studies in which Asian influenza virus (29), Myxovirus para-influenza 1 (30), and a variety of adenovirus types (31) have been used. A comparison of the serologic data from these various studies reveals that the percentage of significant fluorescent-stainable antibody responses approaches those of para-influenza 1 neutralization and Asian influenza virus hemagglutination inhibition; it is in excess of the percentages of complement-fixation responses illustrated.

An association between fluorescent-stainable

TABLE VII

Comparison of the primary atypical pneumonia transmission experiments to several other volunteer studies

Agents employed	Ref.	Volunteers	Volunteers developing illness*		Volunteers' serologic responses (4-fold or >)		
					Test		
		no.	*no.*	*%*		*no.*	*%*
? Eaton agent	(16)	42	31	73.8	Fluorescent-stainable antibody	28	66.6
					Cold hemagglutinins	18	42.8
Asian influenza	(28)	23	18	78.3	Hemagglutination-inhibition	16	69.6
Para-influenza 1	(29)	32	18	56.3	Virus neutralization	22	68.8
					Hemagglutination-inhibition	18	56.3
					Complement fixation	14	43.8
Adenovirus types	(30)						
1, 4 and 5		91	55	60.4	Complement fixation	45	49.5
1, 2 and 3		168	73	43.5	Complement fixation	72	42.9
1–6		100	38	38.0	Complement fixation	36	52.9

* Clinical illness according to the criteria of the study concerned.

antibody responses to Eaton's agent and some cases of primary atypical pneumonia has been established (5–7). The specificity of the reaction is suggested by its absence in limited series of tests using sera from patients with psittacosis, Q fever, adenovirus infections, influenza, and para-influenza 1 and 3 infections (5, 7). The incidence of fluorescent-stainable antibodies in the general population appears to be much greater, however, than the occurrence of primary atypical pneumonia. Figures as high as 40 per cent positive reactions in collections of sera have been reported by Cook (6) and Chanock (7) and their co-workers. In the small sampling of adult males in 1944 that has been presented, 17 of the 70 men (24.3 per cent) had pre-existing antibody titers of 10 or greater. If the Eaton agent indeed played a role in the volunteer experiments analyzed above, it appears that it may also be associated with an undifferentiated minor respiratory illness and with subclinical disease, which offers an explanation for the apparently widespread occurrence of these fluorescent-stainable antibodies. That a spectrum of disease may be associated with the agent(s) of primary atypical pneumonia has been suggested in epidemiologic studies performed by a number of investigators (26, 32–35).

SUMMARY

Preserved sera from 70 volunteers participating in two primary atypical pneumonia transmission experiments were examined for fluorescent-stainable antibodies to the Eaton agent. Fluorescent-stainable antibody responses were associated with cases of primary atypical pneumonia and through a spectrum of disease ranging down to apparent subclinical infection. In contrast, rises of cold hemagglutinins and streptococcus MG agglutinins were limited for the most part to patients with atypical pneumonia and were influenced by the severity of the disease. The explanation of fluorescent-stainable antibody responses in those who had no illness or had minor respiratory illness is not clear; they may represent unapparent or mild infection with the Eaton agent, or they may reflect nonspecific factors as yet unknown. It is concluded that the data presented indicate an association of the Eaton agent with disease in the volunteers studied, although the identity of the agent transmitted could not be determined directly. These results cannot be interpreted as proof of the etiological role of the Eaton agent in primary atypical pneumonia until the exact specificity of the reaction between the agent and fluorescent-stainable antibodies has been established.

ACKNOWLEDGMENT

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THE PLASMA REMOVAL OF INDOCYANINE GREEN AND SULFOBROMOPHTHALEIN: EFFECT OF DOSAGE AND BLOCKING AGENTS

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In 1950 Brauer and Pessotti (1) observed that the concentration of sulfobromophthalein in bile reached maximal values which were not influenced by further increments in infusion rate. In 1956 Combes, Wheeler, Childs and Bradley (2) introduced the concept of maximal biliary transport capacity for sulfobromophthalein. It is now generally held that when sulfobromophthalein is infused at a rate that exceeds the maximal biliary transport capacity, the biliary excretion of the dye remains relatively constant.

The effect of the initial dosage or infusion rate of sulfobromophthalein on the various parameters measuring hepatic uptake is not so uniformly agreed upon, due in part to confusion about the meaning of the parameters themselves. These include: *1*) hepatic extraction—the percentage of dye removed in one passage through the liver; *2*) clearance rate—the milliliters of plasma completely cleared of dye in 1 minute; and *3*) disappearance rate or removal rate—herein defined as the percentage of remaining dye removed each minute. Unless specified, removal rate will not be used to express the number of milligrams removed per minute.

Casselman and Rappaport (3), Andrews, Maegraith and Richards (4) and Combes and associates (2) have suggested that hepatic extraction is constant or nearly constant, regardless of dosage or infusion rate. Myers (5) and Cook, Lawler, Calvin and Green (6) observed the exact converse; that is, that extraction became less efficient with increasing dose. Lavers and colleagues (7) found that variation in the initial single dose did not affect the rate of removal of sulfobromophthalein from the plasma. Again, the converse has been reported: Fauvert (8) stated that increasing the initial dose decreased the plasma disappearance rate of the dye.

Wheeler and associates (9, 10) recently described the hepatic uptake of sulfobromophthalein under special circumstances in which a constant infusion of dye is in excess of the maximal biliary transport capacity. Under these circumstances a portion of plasma removal of the dye is accounted for by bile excretion; this is a constant. The remainder of the dye taken up by the liver from the plasma is stored in the hepatic cells. These authors have shown that hepatic storage is directly proportional to plasma concentration. Consequently, the higher the plasma concentration, the more milligrams of dye can be stored. Limitations were not observed for any potential maximal capacity for this removal mechanism, but it should be noted that the phenomenon is not rate limited. This description does not apply to the mechanics of removal of a single injection of dye, however, because the storage compartment is empty at the outset and, obviously, no constant proportional relationship between storage and plasma concentration would be possible.

Several authors (1, 11–13) reported that sodium dehydrocholate will slow the removal of sulfobromophthalein from the plasma and will delay its excretion in the bile. Cantarow, Wirts, Snape and Miller (14) and Fauvert (8) suggested that competition for hepatic uptake exists between bilirubin and sulfobromophthalein, the latter being selectively removed. Brauer and Pessotti (1) observed that rose bengal slowed the excretion of sulfobromophthalein in bile, but Popper and Schaffner (15) and Fauvert (8) pointed out that sulfobromophthalein is selectively taken up by the liver when administered simultaneously with rose bengal. Wheeler, Cranston and Meltzer (16) reported that the hepatic uptake of sulfobromophthalein was faster than that of indocyanine green; Cherrick, Stein, Leevy and Davidson (17) reported the converse. However, the last two reports were done on different species with different dosage relationships, and the dyes were not administered simultaneously.

In recent studies of hepatic function, indocyanine green has demonstrated the following advantages over other protein-binding dyes: essentially complete recovery in bile (16, 18–20), single exponential plasma removal pattern (16, 17, 19–23), distribution limited to the intravascular volume (16, 24), no demonstrable uptake by extrahepatic tissues (18), no urinary excretion (18–20, 23), no enterohepatic circulation (16), and no reabsorption in the lymphatic circulation (19, 20). Because of these advantages, indocyanine green was employed in these studies in an attempt to clarify the various phenomena and to resolve some of the apparent conflicts.

METHODS

The technic used for the determination of the concentration of indocyanine green in plasma has been described elsewhere (19). Briefly, it consists of measurement of transmittance of light in a spectrophotometer at 805 mμ after appropriate dilution of the specimen.

The concentration of sulfobromophthalein in the plasma was determined by measurement of light transmittance in a spectrophotometer at 580 mμ after dilution and alkalinization. Turbid or hemolyzed specimens were not used.

Determination of the concentration of indocyanine green and sulfobromophthalein in the same specimens of plasma with simultaneous administration of both dyes was performed as follows. Transmittance of light at 805 mμ was measured at neutral pH which thus measured only the indocyanine green, the sulfobromophthalein being decolorized. Then transmittance was measured in the same specimens at 580 mμ after alkalinization. This measured the concentration of sulfobromophthalein, since at this wave length and in this concentration indocyanine green, while not decolorized, transmits 98 to 100 per cent of light (25). In these studies the dosage of sulfobromophthalein was five times greater than that of indocyanine green in order that the indocyanine green be sufficiently dilute not to interfere with transmittance at 580 mμ, and that no intervening dilutions be required between measurements, since indocyanine green absorbs light more intensively than does sulfobromophthalein.

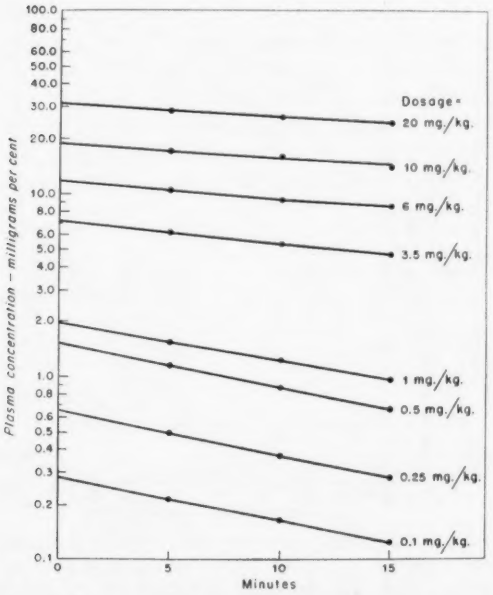

FIG. 1. PLASMA DISAPPEARANCE RATE OF INDOCYANINE GREEN IN DOG A.

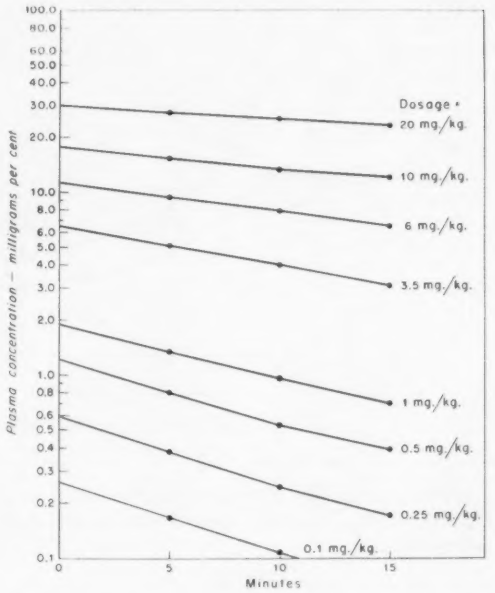

FIG. 2. PLASMA DISAPPEARANCE RATE OF INDOCYANINE GREEN IN DOG B.

The method of calculation of the plasma disappearance rate has been described previously (8, 19). We have found (19, 20), as have others (16, 18), that the plasma disappearance rate of indocyanine green in dogs is exponential for at least 15 minutes, and usually for 30 to 60 minutes, in essentially all cases. Since the present studies of dose effect and blocking are concerned with phenomena occurring in the early phase of removal from the plasma, blood samples were taken at 5, 10 and 15 minutes after injection of dye.

The plasma removal of sulfobromophthalein in dogs and rats does not follow a single exponential pattern, but for description of the phenomena in the early phase of removal, a useful approximation can be reached by taking two points on the early portion of the disappearance curve and substituting these values in the equation as if the

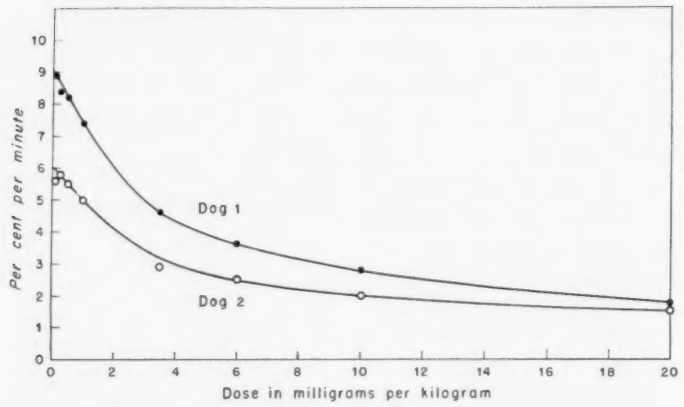

FIG. 3. THE DOSE AND PLASMA DISAPPEARANCE RATE ARE RELATED INVERSELY; LARGER DOSES HAVE PROPORTIONATELY SMALLER EFFECTS.

pattern were exponential. The removal rates of sulfobromophthalein in this study were so obtained and apply only to this time.

RESULTS

The plasma disappearance rate of indocyanine green [1] was determined in each of two unanesthetized dogs on eight separate occasions with doses of dye which varied from 0.1 to 20.0 mg per kg body weight. Blood was sampled at 5, 10 and 15 minutes. The removal curves were consistently exponential for the 15-minute observation (Figures 1 and 2). As the initial dose increased, the plasma disappearance rate decreased but not in linear fashion. The apparent similarity of the slopes of the lines in Figures 1 and 2 is the result of the scale including 3 log cycles. The larger dosage of dye exerted a proportionately smaller slowing effect (Figure 3).

When the plasma disappearance rate was multiplied by the initial dose (per cent per minute times milligrams per kilogram), an expression of the actual number of milligrams per minute removed in the initial moments was obtained (milligrams per minute per kilogram). This figure applies only to the initial moments because, with a constant percentage being removed (exponential rate), the milligrams per minute will decrease steadily with time.

When this initial removal rate in milligrams per minute per kilogram was plotted logarithmically against the initial dose (Figure 4), it was seen that with larger doses a larger amount of dye was removed, even though the percentage was smaller. Logarithmic plots were made of the difference between the observed removal rate and various arbitrary possible asymptotes to the curve in Figure 4. With 0.4 mg per kg weight per minute

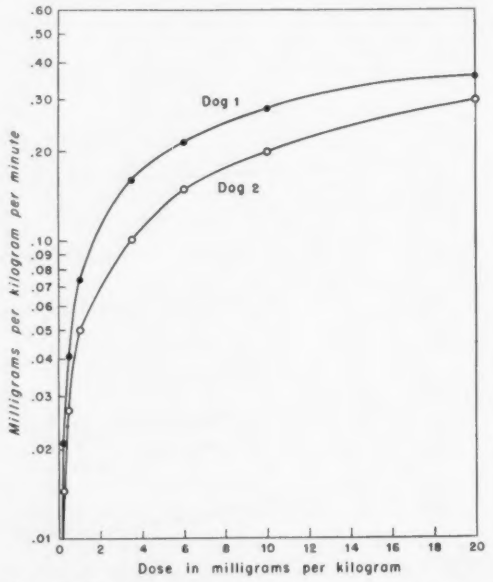

FIG. 4. THE REMOVAL RATE IN MILLIGRAMS PER MINUTE PER KILOGRAM INCREASES WITH INCREASING DOSAGE BUT AN ASYMPTOTE IS APPROACHED AT 0.4 MG PER MINUTE PER KG, WHICH REPRESENTS THE MAXIMAL CAPACITY FOR REMOVAL OF DYE.

[1] Supplied as cardio-green through the courtesy of Hynson, Westcott & Dunning Pharmaceutical Laboratories, Baltimore, Md.

as an asymptote, the log plot of the difference was a straight line. This suggests that the asymptotic value is the maximal initial (rate limited) removal capacity.

The initial plasma disappearance rate of sulfobromophthalein was estimated in two unanesthetized dogs. Blood was sampled at 5, 10 and 15 minutes, and the 5-minute and 10-minute values were substituted in the equation as described. Initial doses of 3.3 and 33.0 mg of sulfobromophthalein per kg body weight were used in separate tests. The initial plasma disappearance rate (Figure 5) of Dog C decreased from 18.6 to 13.6 per cent per minute when dosage of dye was increased tenfold; the plasma disappearance rate of Dog D decreased from 20.4 to 12.6 per cent per minute. Thus, sulfobromophthalein removal rates in dogs were decreased by increasing the dosage.

Two unanesthetized dogs were subjected to a series of tests in which various agents were injected simultaneously with a standard dose of indocyanine green to test the effect of each agent on the plasma disappearance rate of the indocyanine green. One mg of indocyanine green equals 0.0013 mmole. In each case the dose of the blocking agent, that is, sulfobromophthalein, rose bengal, bilirubin, sodium dehydrocholate, or indocyanine green itself, was molecularly 10 times the standard dose of indocyanine green or 0.013 mmole (Table I). Bilirubin also was given in twice the molecular dose, or 0.0026 mmole. Blood was sampled at 5, 10 and 15 minutes and revealed exponential removal patterns (Dog B, Figure 6).

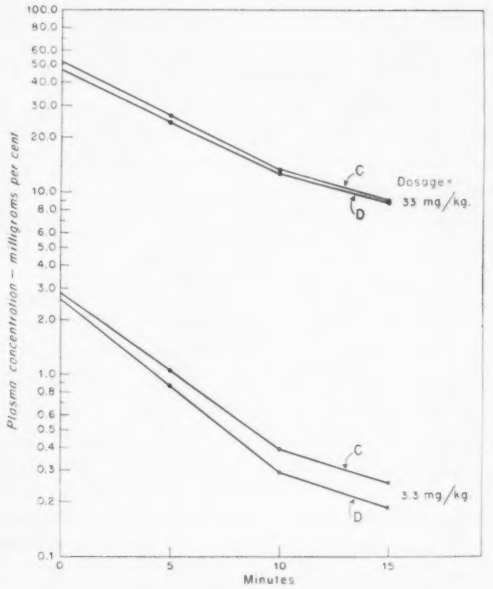

FIG. 5. THE PLASMA DISAPPEARANCE RATE OF SULFOBROMOPHTHALEIN IN DOGS IS NOT EXPONENTIAL. The initial rate is estimated from the early portion of the curve. Rates are shown for Dogs C and D.

The extent to which sulfobromophthalein, rose bengal, bilirubin, and indocyanine green itself, slowed the removal rate of the indocyanine green was the same when equivalent doses of these blocking agents were used (Figure 6, Table I). The effect of bilirubin in a dose of 0.0026 mmole was significantly less than the effect of the larger amount. Bilirubin was dissolved in 0.1 M sodium

TABLE I

Effect of blocking drugs on removal of indocyanine green

Drug	Formula		Dose	Rate	
				Dog A	Dog B
		mol wt	*mg/kg*	*%/min*	
Indocyanine green	$C_{43}H_{47}O_6N_2S_2Na$	775	1.0*	4.5–5.0	6.1–7.4
			11.0†	3.3	4.0
Sulfobromophthalein	$C_{20}H_8Br_4Na_2O_{10}S_2$	838	10.9	2.8	4.1
Rose bengal	$C_{20}H_2O_5I_4Cl_4Na_2$	1,018	13.2	3.3	4.0
Bilirubin	$C_{32}H_{36}N_4O_6$	573	7.4	2.2	3.6
			1.5	3.1	5.8
Sodium dehydrocholate	$C_{24}H_{33}NaO_5$	415	5.4	4.5	7.1

* Standard dose of indocyanine green.
† Standard dose of indocyanine green plus the tenfold blocking dose.

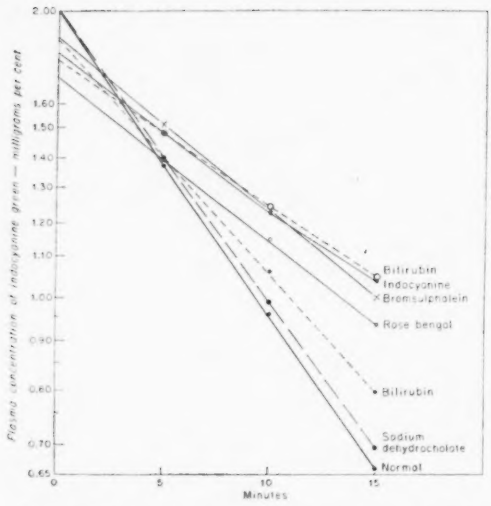

FIG. 6. THE REMOVAL RATES OF INDOCYANINE GREEN WITH THE VARIOUS BLOCKING DRUGS ARE EXPONENTIAL. Similarity of removal rate is denoted by similarity of slope. The slower slope applies to the larger dose of bilirubin. Data for Dog A are not illustrated but are similar to those shown here for Dog B.

carbonate for intravenous administration. The same amount of 0.1 M sodium carbonate given alone had no effect on the plasma disappearance rate of indocyanine green. Sodium dehydrocholate, when given in the same molecular dose of 0.013 mmole, did not produce a measurable decrease in the plasma disappearance rate of indocyanine green (Table I); but, when given in a much larger dose of approximately 260 mg per kg body weight (10 ml of a 20 per cent solution), it produced definite slowing of the plasma disappearance rate (1.2 and 2.1 per cent per minute in the two dogs).

The initial plasma disappearance rate of sulfobromophthalein was measured in two unanesthetized dogs by giving an initial dose of 5 mg of sulfobromophthalein per kg body weight and by sampling blood at 5, 10 and 15 minutes. The removal rates were not exponential, but the initial estimated plasma disappearance rates were 23.9 and 21.0 per cent per minute, respectively. Then 1.0 mg of indocyanine green per kg body weight was administered simultaneously with the standard dose of 5 mg sulfobromophthalein per kg body weight. Under these circumstances the estimated initial plasma disappearance of sulfobromophthalein in the two dogs was 14.8 and 16.9 per cent per minute, respectively (average of three tests in each dog). Thus it was seen that indocyanine green could slow or block the removal rate of sulfobromophthalein.

The initial plasma disappearance rate of sulfobromophthalein was determined in four Sprague-Dawley rats weighing approximately 250 g. The rats were under ether anesthesia, and cardiac blood was sampled 3, 6 and 9 minutes after injection of 5 mg sulfobromophthalein (approximately 20 mg per kg body weight; Table II). The plasma removal pattern was not exponential, but the initial plasma disappearance rates taken from specimens withdrawn at 3 and 6 minutes after injection averaged 35.6 per cent per minute.

Next, the plasma disappearance rate of indocyanine green was determined in six Sprague-Dawley rats weighing approximately 250 g under the above mentioned conditions after injection of 1.0 mg indocyanine green (approximately 4 mg per kg body weight). The results (Table II) revealed nonexponential removal rates (19) averaging 34.0 per cent per minute in the initial 6 minutes. Then 1.0 mg indocyanine green and 5 mg sulfobromophthalein were given simultaneously to four Sprague-Dawley rats weighing ap-

TABLE II

Plasma disappearance rates of indocyanine green and sulfobromophthalein in Sprague-Dawley rats

Dog	Indocyanine green		Sulfobromophthalein	
	Dose	Rate	Dose	Rate
	mg	*%/min*	*mg*	*%/min*
1	1.0	26.7		
2	1.0	30.1		
3	1.0	31.5		
4	1.0	31.5		
5	1.0	40.8		
6	1.0	43.3		
Average (1–6)		34.0		
7			5.0	30.1
8			5.0	33.0
9			5.0	38.5
10			5.0	40.8
Average (7–10)				35.6
11	1.0	11.2	5.0	21.0
12	1.0	14.1	5.0	25.7
13	1.0	16.1	5.0	36.5
14	1.0	19.2	5.0	30.1
Average (11–14)		15.1		28.3

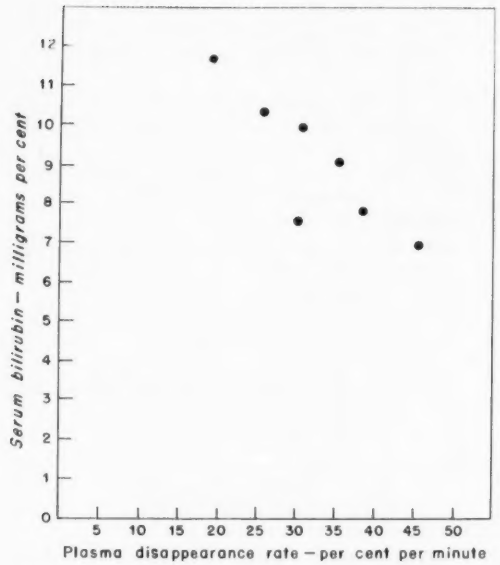

FIG. 7. PLASMA REMOVAL OF INDOCYANINE GREEN IN GUNN RATS. With one exception the points form a nearly straight line, indicating an inverse linear relationship between plasma disappearance rate of indocyanine green and the total serum bilirubin.

proximately 250 g each, and the removal rate of each dye was determined as before. The results (Table II) indicate that the removal rate of each dye was decreased. The amount of slowing was more prominent with indocyanine green, because in this instance the dose of the blocking dye, sulfobromophthalein, was five times greater than the dose of the test dye. Conversely, the slowing with sulfobromophthalein was less pronounced, but here the blocking dye, indocyanine green, was given in only one-fifth of the dose of the test dye.

It had been reported previously by the authors (20) that the plasma disappearance rate of indocyanine green in a group of congenitally jaundiced Gunn rats was not statistically different from the plasma disappearance rate in normal Sprague-Dawley rats. However, it had been noted that the range of removal rates was wide and so an attempt was made to compare the plasma disappearance rate with the degree of jaundice. The plasma disappearance rate of indocyanine green, therefore, was determined in seven Gunn rats weighing approximately 250 g, with the rat under ether anesthesia during sampling of cardiac blood after administration of 1.0 mg of indocyanine green. Total serum bilirubin was measured by the Malloy-Evelyn method (Figure 7). An approximately inverse linear relationship exists between the plasma disappearance rate and the serum bilirubin. The group was too small for the reliable application of statistical methods permitting more precise description of the relationship.

CONCLUSIONS

These studies confirm the previous observation that the plasma disappearance rate of sulfobromophthalein is slower with large initial doses than with small doses of the dye. A similar phenomenon has been demonstrated for the plasma disappearance of indocyanine green. It has been shown further that the relationship between dye dosage of indocyanine green and the plasma disappearance rate is a nonlinear inverse one. It would therefore appear that, when removal rates of one dye are compared with those of another (16, 17), the comparison must be qualified for the dose used. However, these studies did indicate that, milligram for milligram or millimole for millimole, sulfobromophthalein was more rapidly removed from dog and rat plasma in the initial phase than was indocyanine green. If it is kept in mind that the present study is limited to the removal of a single dose and that the storage compartment is unsaturated at the outset, it appears that the capacity of the liver to take up indocyanine green from the plasma of dogs has a maximal rate limit (Figure 4).

Since smaller initial doses are more quickly removed from the plasma, these studies also seem to indicate that dye already absorbed by the liver inhibits subsequent absorption of dye. Were this not the case, as the plasma concentration of dye decreases with time, the removal rate of the smaller remaining amount should increase rather than continue at the same rate. Previous studies (19, 20) indicate that indocyanine green is not reabsorbed into the hepatic lymph, and hence it appears unlikely that reabsorption would explain this observation. These additional observations on uptake of dye by the liver in no way disagree with the phenomena described by Wheeler and his colleagues (9, 10).

These studies do not appear to confirm the con-

cept of *a priori* selective uptake of some dyes during simultaneous administration. All of the dyes studied exert the same blocking effect when given in equimolecular doses, and it has been shown that sulfobromophthalein and indocyanine green show mutual blocking. The blocking effect seems to be molecular competitive inhibition, since a single agent, indocyanine green, blocks itself similarly to the other blocking dyes. The blocking produced by sodium dehydrocholate is less pronounced, and its nature is not clarified by these studies. The demonstration that bilirubin blocks the removal of indocyanine green in dogs, and that the plasma disappearance rate is related inversely to the degree of jaundice in Gunn rats, is in accord with an observation made earlier (19), that obstructive jaundice in human beings produces an inverse linear slowing effect on the plasma disappearance rate of indocyanine green.

SUMMARY

It has been shown that the removal of indocyanine green from dog plasma is an inverse function of the initial single dose. A maximal initial transfer capacity for hepatic uptake after a single dose of dye has been found.

The mechanism whereby several dyes, taken up by the liver, compete with each other or block each other appears to be molecular competitive inhibition. The data do not support the concept that some of these dyes are, *a priori*, taken up selectively.

It has been shown that bilirubin blocks the removal of indocyanine green as do the dyes, and that the rate of removal of indocyanine green in Gunn rats is dependent on the degree of bilirubinemia.

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AN IMMUNOLOGIC BASIS FOR ACQUIRED RESISTANCE TO ORAL ADMINISTRATION OF HOG INTRINSIC FACTOR AND VITAMIN B_{12} IN PERNICIOUS ANEMIA *

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In pernicious anemia, vitamin B_{12} deficiency develops owing to the failure of gastric intrinsic-factor secretion. Patients with this disease absorb vitamin B_{12} from the gastrointestinal tract when it is fed together with an extract of hog pyloric mucosa. Some patients have relapsed during the course of oral therapy with this mixture (1–3). These subjects absorb vitamin B_{12} administered with normal human gastric juice but do not absorb it when it is administered with hog preparations possessing intrinsic-factor activity (3). These patients have become refractory to the hog preparations. It has been reported that sera obtained from most of these refractory patients (4), and from a minority of nonrefractory patients and normal subjects (5, 6), possess intrinsic factor-neutralizing properties.

Such observations have directed attention to a possible immunologic mechanism for the refractory state. This study was undertaken to determine whether the refractory state might be due to antibodies against the hog intrinsic-factor preparation, which could be detected *in vitro*.

METHODS AND MATERIALS

The standard hog pyloric mucosal extract [1] (hog IF) was active in the Schilling test at a dosage of 100 mg.

Normal human gastric juice was obtained from at least 4 donors after histamine stimulation. After filtration through cheesecloth the juice was adjusted to pH 10.0 with 2 N sodium hydroxide for 20 minutes to inactivate pepsin (7), neutralized to pH 7.0 to 8.0 with 2 N hydrochloric acid, and stored at −20° C.

To determine whether intrinsic factor could be bound by diazonium groups, fixed diazonium groups were prepared by exposing a column of polyaminopolystyrene to nitrous acid, as described by Gyenes, Rose and Sehon (8). After one or more passages through this column, hog pyloric extract was tested for intrinsic-factor activity *in vivo* by the Schilling test.

Serum was tested for antibodies by the technique of Stavitsky and Arquilla (9), which uses *bis*-diazotized benzidine to coat rabbit erythrocytes with antigen. Agglutination of these coated erythrocytes by dilutions of serum was considered to be due to antibodies' combining with the hog proteins bound to the erythrocytes. The specificity of the antibodies was determined by absorbing the serum with hog pyloric extracts, or with nonpyloric hog protein before addition of the coated erythrocytes. Specific agglutination of the coated erythrocytes was defined as agglutination which could be prevented by absorption of the antiserum with hog pyloric mucosal extracts.

To coat the erythrocytes, *bis*-diazotized benzidine solution [2] was mixed with a 10 per cent (wt/vol) aqueous solution of hog IF, in a total volume of 1.5 ml. To this was added 1.2 ml of a 25 per cent suspension of washed rabbit erythrocytes in normal saline. The optimal ratio of benzidine to antigen was determined previously by carrying out the procedure with different proportions of these materials. With the reagents described here, approximately 1 ml of antigen and 1 ml of the benzidine solution provided optimal coating of the erythrocytes.

The mixture was incubated at room temperature for 10 minutes, agitated, and centrifuged at about 1,200 G. The cells were washed in 3.5 ml of diluent (0.15 M phosphate buffer at pH 7.3 containing 1 per cent of heat-inactivated normal rabbit serum) and suspended in 2.5 ml of the diluent.

Antiserum was incubated at 56° C for 30 minutes to inactivate complement, and diluted serially in 0.5 ml volume of the diluent. All sera were diluted in triplicate, and 0.05 ml of the coated cells was added to each tube of two series of dilutions. In one of these series, 0.1 ml of

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[1] Ayerst 5310 defatted, desiccated hog pyloric mucosa, generously supplied by Ayerst McKenna & Harrison Ltd., Montreal.

[2] *Bis*-diazotized benzidine was prepared by slowly adding 5 ml of 8.75 per cent (wt/vol) sodium nitrite ($NaNO_2$) to 0.23 g benzidine in approximately 10 ml water at 0° C. Water was added to 50 ml. Aliquots were frozen in an alcohol-dry ice mixture, and stored at −20° C; 1 ml of this solution diluted in 14 ml of 0.15 M phosphate buffer (pH 7.3) was used in the coating procedure.

antigen solution was incubated with each dilution for 15 minutes before adding the coated cells. A control was set up by adding uncoated cells to the third series of dilutions.

The test was read after 5 hours' incubation at room temperature. Hemagglutination was recognized by the pattern of sedimentation of erythrocytes at the bottom of the test tubes. Unagglutinated erythrocytes sedimented in a button or ring, whereas agglutinated cells settled in a homogeneous layer.

After the agglutination pattern was recorded, 0.1 ml of 5 per cent PVP [3] in phosphate buffer was added to each tube. The tubes were agitated, incubated, and the pattern of settling again noted. This procedure increased the sensitivity of the test.

Rabbits, mixed as to sex and breed, were immunized by the intravenous injection of 3 mg of the standard hog IF preparation in saline. Injections were carried out twice weekly for 3 months, separated by rest periods of 1 month. After two such periods, the serum was assayed for antibodies.

Vitamin B_{12} absorption from the gastrointestinal tract in man was determined by means of the Schilling test. The dose of Co^{58}-vitamin B_{12} [4] administered orally was 0.5 μg (specific activity approximately 2 μc per μg) and the "flushing" dose of 1,000 μg of nonradioactive vitamin B_{12} was administered intramuscularly 2 hours after the oral dose. Urine collections were made for at least 24 hours, and usually for a second 24-hour period at the beginning of which a second injection of 1,000 μg of vitamin B_{12} was given. The collection of urine was checked by determining the 24-hour urinary creatinine excretion. The test described by Schwartz, Lous and Meulengracht (4) for anti-intrinsic factor substances in serum was performed by feeding 15 ml of rabbit serum or 30 ml of human serum to the subject immediately before oral administration of an aqueous mixture of hog IF and Co^{58}-vitamin B_{12}.

To study the electrophoretic mobility [5] of intrinsic factor, 0.25 μg of Co^{58}-vitamin B_{12} and 10 mg of the hog pyloric extract were dialyzed against water at 4° C for 24 hours. The dialyzed material was mixed with serum from immunized and nonimmunized rabbits, and electrophoresed on Whatman no. 3 paper by a modification (10) of the technique of Grassmann and Hannig (11). The strips obtained were cut into segments and the radioactivity determined in a well-type scintillation counter. Longitudinal segments of the strips were stained for protein with Amidoschwartz 10 B.

Precipitins were sought by a standard saline precipitin technique, with hog IF to which a small quantity of Co^{58}-vitamin B_{12} had been bound (12). The precipitates were washed in 0.85 per cent saline, and radioactivity determined.

[3] Polyvinylpyrrolidone, K value 60, molecular weight 160,000, generously supplied by Antara Chemicals Corp., Montreal.

[4] Obtained from Merck & Co., Ltd., Montreal.

[5] Electrophoresis performed through the cooperation of Dr. B. Rose and Dr. E. McGarry, Royal Victoria Hospital, Montreal.

TABLE I

Schilling tests with hog intrinsic factor (IF) previously exposed to diazonium groups

Substance administered	Passages through column	Radioactivity in urine
	no.	%
B_{12}	0	1.3
B_{12} + IF	0	16.2
B_{12} + IF	1	5.4
B_{12} + IF	2	1.7

OBSERVATIONS

A solution containing 200 mg of the standard hog IF preparation was passed through the diazotized polyaminopolystyrene column. The intrinsic-factor activity of this preparation was markedly reduced by passage through the column and was completely abolished by a second passage (Table I), thus demonstrating that hog intrinsic factor is bound by diazonium groups.

Rabbit serum studies. Dilutions of sera, obtained from immunized rabbits, agglutinated erythrocytes coated with hog IF (Figure 1). Uncoated erythrocytes, or erythrocytes coated with beef or hog insulin by the same technique, were not agglutinated. The antibody titer was abol-

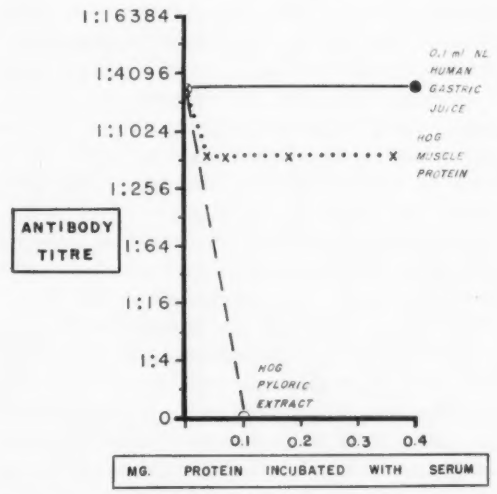

FIG. 1. THE EFFECT ON THE HEMAGGLUTINATION TITER OF PRIOR ABSORPTION OF RABBIT ANTISERUM WITH NORMAL HUMAN GASTRIC JUICE, HOG MUSCLE EXTRACT, AND HOG IF.

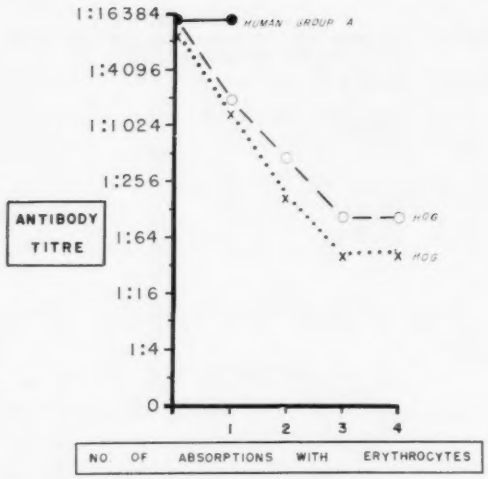

FIG. 2. THE EFFECT ON THE HEMAGGLUTINATION TITER OF PRIOR ABSORPTION OF RABBIT ANTISERUM WITH HOG AND HUMAN ERYTHROCYTES.

ished after incubation of the antiserum with a solution of hog pyloric mucosal extract; it was reduced, but not abolished, after absorption with a soluble extract of hog muscle. Repeated absorptions of the rabbit antiserum with hog erythrocytes reduced but did not abolish the titer (Figure 2). Absorption of antiserum with normal human gastric juice previously dialyzed against water did not alter the titer. In each titration, the coated cells also were exposed to serum from nonimmunized rabbits. These sera did not agglutinate the coated cells. Several hog gastric extracts,[6] prepared by various techniques such as differential solubility, dialysis, and electrophoresis, were tested for blocking by this technique.

Antiserum absorbed with each of these preparations was exposed to erythrocytes coated with the standard hog IF. The preparations with IF activity, as determined by the Schilling test, completely blocked the agglutination of the coated cells (data not shown). Addition of the hog muscle extract to a potent antiserum reduced the titer from 1:10,000 to 1:640 (Table II), but subsequent absorption of this antiserum with hog erythrocytes did not further reduce the titer. The

[6] Generously supplied by Dr. F. C. McIntire, Abbott Labs., North Chicago, Ill.; and Dr. L. Ellenbogen, Lederle Labs., Pearl River, N. Y.

TABLE II

Absorption of rabbit antiserum with hog muscle extract, hog erythrocytes, and hog IF; effect on hemagglutination titer

Treatment of antiserum			
Addition of			
Hog IF	Hog muscle extract	Absorption with hog RBC's	Titer of serum
−	−	−	1:10,240
+	−	−	0
−	+	−	1:640
−	+	+	1:640
+	+	+	0

titer was abolished by absorption of the antiserum with soluble hog IF. Absorption of the antiserum with five other hog pyloric preparations which were effective in promoting the absorption of Co^{58}-vitamin B_{12} in nonrefractory patients, in doses of 0.5 to 25 mg, abolished this residual titer. One hog gastric preparation, which was devoid of intrinsic-factor activity, did not abolish this titer. The titer which persisted after absorption with hog muscle or erythrocytes, and which subsequently was absorbed only by hog pyloric extracts possessing intrinsic-factor activity, was considered as probably representing antibodies to hog intrinsic factor.

Human serum studies. The sera of six patients who had become resistant to oral therapy while receiving a hog IF preparation were tested by this technique. The titer of the antibodies found,

TABLE III

Refractory patients; antibody titers and urinary excretion of Co^{58}-vitamin B_{12} (per cent of oral dose) *

Patient	Titer	Schilling test: Intrinsic factor administered with vitamin B_{12}: None	Hog IF	NHGJ
LW	1:100	1.9	<0.1	14.6
WS	1:16	<0.1	0.2	11.1
PC	1:4	1.4	<0.1	†
HT	1:2	<0.1	0.5	16.0
FC	1:2	2.1	4.7	21.2
SS	0	<0.1	4.3	13.5

* The data shown correlate the *in vitro* titer of hemagglutinating antibodies with the results of Schilling tests in six refractory patients. The excretion values represent the % dose of Co^{58}-vitamin B_{12} excreted in the urine when the B_{12} was administered alone, with hog pyloric extract (hog IF) and with 40 ml of neutralized normal human gastric juice (NHGJ).

† At inception of therapy, patient responded optimally to oral vitamin B_{12} and hog IF.

together with results of Schilling tests, are shown in Table III. The titers described represent the titer of hemagglutination that could be abolished by prior absorption of the serum with hog IF. Antibodies were present in five patients but at a high titer in only one. The immunologic properties of the antibodies found in the serum of the patients with a titer of 1:100 are summarized in Figure 3. Other sera gave the same pattern of immunologic properties. The antibodies were neutralized by the addition of soluble hog IF (Figure 3) but were not affected by absorption with soluble hog muscle extract or with normal human gastric juice. In each hemagglutination experiment, antibodies were sought in serum obtained from patients with pernicious anemia who had not become refractory to oral medication, or who had been treated only with injections of vitamin B_{12}. Sera from 20 such patients were tested. No hemagglutinating antibodies were found in any of these sera, nor was any detected in sera obtained from 10 normal subjects.

Other immunologic procedures. During electrophoresis of a mixture of normal rabbit serum and hog IF, the vitamin B_{12}-binding protein migrated with the β-globulins (Figure 4). With immune rabbit serum, the B_{12}-binding peak migrated with the γ-globulins. This suggests that the immune rabbit sera contained antibodies that combined with the intrinsic-factor portion of the hog mucosal extract. This effect was noted also after the antiserum had been absorbed with hog muscle.

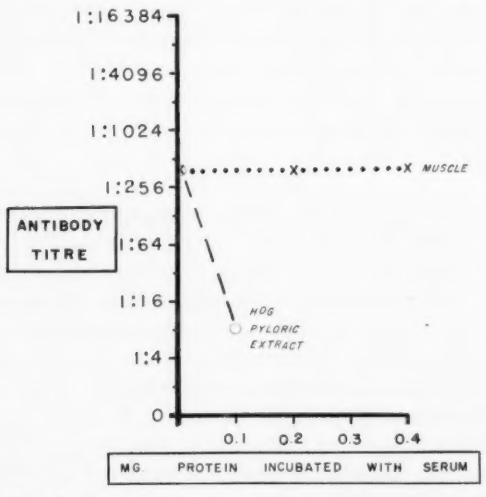

FIG. 3. THE EFFECT ON THE HEMAGGLUTINATION TITER OF PRIOR ABSORPTION OF HUMAN SERUM WITH HOG IF AND HOG MUSCLE EXTRACT. As shown here, high concentrations of human serum occasionally caused agglutination of the coated erythrocytes, which could not be prevented by absorption of the serum with hog pyloric extract. As described in the text, the antibody titer was defined as the titer of hemagglutination that could be prevented by absorption of the serum with hog IF.

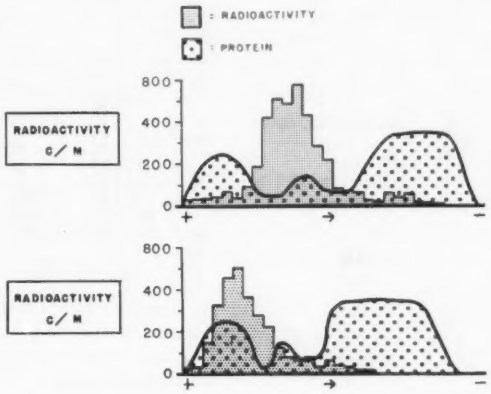

FIG. 4. PAPER ELECTROPHORESIS OF HOG IF WITH NORMAL AND IMMUNE RABBIT SERUM. Co^{58}-vitamin B_{12} bound to hog IF migrated with the β-globulins of normal rabbit serum (*upper*); with immune serum, it migrated with the γ-globulin (*lower*).

When serum from immunized rabbits was mixed with a hog pyloric extract in saline, optimal precipitation occurred at a dilution of 1:2. All of the radioactive vitamin B_{12} added was recovered in the supernatant, suggesting that the B_{12}-binding fraction of the pyloric extract was not involved in the precipitation reaction.

The results of Schilling tests performed on nonrefractory patients with classical pernicious anemia are summarized in Figure 5 and Table IV. These patients were fed rabbit serum and human serum, both normal and immune, together with Co^{58}-vitamin B_{12} and hog IF in various combinations. A rough correlation appears to be present between titers of antibodies present in the human sera and degree of inhibition of absorption of radioactive vitamin B_{12} in the presence of hog IF (Figure 5). A similar rough correlation appears to exist between *in vivo* activity of the rabbit antisera and *in vitro* titer after absorption with hog muscle extract (Table IV). The unabsorbed

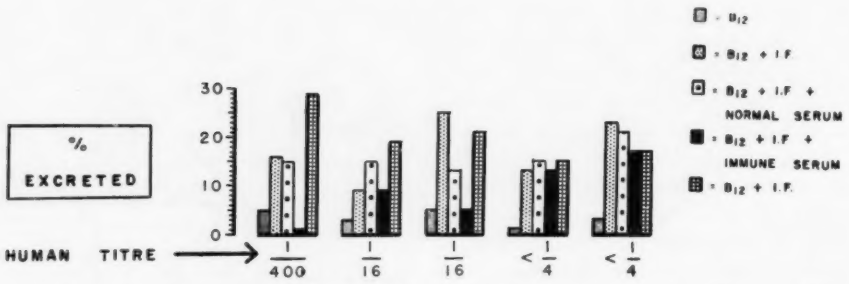

FIG. 5. VITAMIN B_{12} ABSORPTION (SCHILLING) TESTS IN PERNICIOUS ANEMIA PATIENTS: EFFECT OF MIXING NORMAL AND "IMMUNE" HUMAN SERUM WITH HOG IF. Each set of bars represents studies on one nonrefractory patient. Figures beneath the diagrams indicate the titer of antibodies in the immune serum.

titers of rabbit antiserum did not correlate with this *in vivo* activity.

An attempt was made to overcome the refractory state, by the technique described by Schwartz and co-workers (4). A large quantity of hog IF (1,000 mg) was administered to one refractory patient (HT). This resulted in the urinary excretion in 48 hours of 5.6 per cent of the administered radioactivity (Schilling test). Three days later this patient excreted only 0.3 per cent of the radioactivity when the B_{12} was administered with 100 mg of the hog IF preparation. This suggests that the inhibition of B_{12} absorption was overcome partially by an excess of hog IF but that this did not permit the efficient action of a smaller quantity of hog IF 3 days later.

TABLE IV

Sequential Schilling tests in one nonrefractory patient; hog IF and Co^{58}-vitamin B_{12} were administered together with different rabbit antisera *

Schilling test 48-Hour excretion	Rabbit antiserum		
	Rabbit	Total titer	Titer after absorption with hog muscle
%	*no.*		
16.2	none		
10.1	I	1:480	1:120
15.6	II	1:480	1:60
17.0	III	1:120	1:45†
9.0	IV	1:960	1:120
22.2	none		

* The hemagglutination determinations were performed on two different occasions, and both determinations gave similar results. Titers were determined by performing halving dilutions on 1:30 dilutions of serum, with and without prior absorption with soluble hog muscle extract.
† Partial agglutination at 1:30; complete agglutination at 1:60.

Thus, the antibodies detected in the sera of the immunized rabbits were partially absorbed by hog erythrocytes or hog muscle protein, but were completely neutralized only by hog pyloric extracts possessing intrinsic-factor activity. These antibodies combined with the vitamin B_{12}-binding portion of a hog pyloric extract and altered its electrophoretic mobility. The antibodies detected in the sera of five of six patients who were refractory to hog pyloric material were not absorbed by nongastric hog protein or by human gastric juice, but only by hog preparations with intrinsic-factor activity. Sera obtained from nonrefractory patients with pernicious anemia and from normal subjects did not contain such antibodies.

DISCUSSION

The ingestion of partially purified hog pyloric extracts over a prolonged period elicits antibody formation in some subjects. As described in a preliminary report of these studies (13), the antibodies appear to combine with the intrinsic-factor portion of hog pyloric material to render the hog preparations ineffective in promoting vitamin B_{12} absorption. Unlike the antibodies described by others, they do not react with human gastric juice, and this material promotes absorption of vitamin B_{12} in refractory patients.

The precipitins which appear in the sera of rabbits immunized with hog pyloric mucosal extracts appear to precipitate proteins other than those that bind vitamin B_{12}; i.e., intrinsic factor. This may explain the irregular correlation between precipitin titers and *in vivo* activity found by Taylor and Morton (14).

Schwartz and colleagues (4) have postulated hog intrinsic factor-neutralizing antibodies in the sera of refractory patients, because they absorb vitamin B_{12} mixed with human and rat gastric juice, and with massive doses of hog intrinsic factor, but do not absorb B_{12} with wild-boar gastric juice or with small doses of hog intrinsic factor. They have also demonstrated that serum from such refractory patients appears partially to neutralize the activity of hog IF concentrates and human intrinsic-factor preparations when fed, together with such substances, to nonrefractory patients. This test for intrinsic factor-neutralizing substances in the serum is crude, and the results do not always correlate with the refractory state (4–6). Our data suggest that the hemagglutinating antibodies described probably are associated with the refractory state.

Antibody formation to hog intrinsic factor requires penetration of the intact intrinsic-factor protein into the body. Antibody stimulation by orally administered nonliving antigens has been reported (15, 16). In human serum, the absence of antibodies that could be absorbed with nongastric hog protein suggests that it is only the intrinsic-factor portion of the extract that enters the body. The absence of a demonstrable titer of antibodies in the serum of one refractory patient may imply that the antibody is present only in the mucosal cells, or that the circulating antibody titer was too low to be demonstrated by the present technique.

The demonstration by Schwartz and co-workers (4), that the refractory state is uncommon in patients receiving crude preparations of hog pylorus, may be explained by a less ready penetration of the intestinal mucosa by intrinsic factor in the presence of large quantities of other proteins. Another explanation would be to postulate the breakdown of a macromolecule of intrinsic factor into smaller active units which may more rapidly be absorbed intact from the gastrointestinal tract.

Glass, Stephanson, Rich and Laughton (17) have suggested that intrinsic factor may be a small molecule, bound to a larger molecule. In the purification procedures, the bond between the intrinsic factor and its carrier might be weakened, and one or the other molecule might be more readily absorbed into the body to stimulate antibody formation. If this were true, then antibodies against either the intrinsic factor or the carrier protein might inhibit vitamin B_{12} absorption.

The observation that patients treated with crude hog preparations absorb vitamin B_{12}, but usually do not develop antibodies to hog intrinsic factor, suggests that the intrinsic factor does not penetrate the intestinal mucosa in these subjects. This indicates that the vitamin B_{12} absorbed is not necessarily accompanied by an immunologically active portion of the intrinsic factor.

SUMMARY

Antibodies that will react with hog pyloric mucosal extracts have been demonstrated by means of a hemagglutination technique. These antibodies do not react with normal human gastric juice, hog erythrocytes, or hog muscle protein. They have been found in sera obtained from rabbits immunized with an extract of hog pyloric mucosa, and in sera from certain patients with pernicious anemia who had become resistant to oral therapy with hog gastric extracts, but they have not been found in nonrefractory patients or in normal subjects.

Human and rabbit sera containing these antibodies, when mixed with hog IF and vitamin B_{12} and fed to nonrefractory patients with pernicious anemia, inhibited the absorption of vitamin B_{12} to a degree approximately proportional to the *in vitro* antibody titer.

The properties of these antibodies are consistent with the hypothesis that they may be responsible for acquired resistance to oral therapy with hog gastric material in pernicious anemia.

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THE BINDING OF ALDOSTERONE TO PLASMA PROTEINS IN NORMAL, PREGNANT, AND STEROID-TREATED WOMEN *

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There is now abundant evidence that both cortisol and corticosterone are bound in plasma to a protein with high affinity and low capacity (probably an α-globulin termed transcortin) as well as to albumin (1–10). It has also been shown that either the concentrations of transcortin or its affinity for cortisol is increased in pregnancy and after estrogen treatment (4, 10), although one group (10) has not found increased binding of tracer amounts of cortisol in pregnancy. Preliminary studies by Mills, Chen and Bartter (7, 8) indicated that aldosterone is bound to proteins other than albumin to a much lesser extent than is cortisol. Sandberg, Slaunwhite and Carter (9) reported an appreciable binding of aldosterone in diluted plasma dialyzed against albumin at 4° C. The aldosterone was readily displaced from the binding protein by the addition of cortisol, while cortisol binding under similar conditions was not significantly reduced by the addition of physiological amounts of aldosterone (9). During the preparation of this manuscript Daughaday, Holloszy and Mariz (10) reported that at 37° C they could not demonstrate any binding of aldosterone to proteins other than albumin in the plasma of normal women or in that of pregnant subjects and women treated with estrogen, but they found appreciable binding of aldosterone to such a protein in normal plasma at 4° C. The present paper gives detailed data on the binding of aldosterone in normal plasma, obtained by a microdialysis technique with 7-H^3-aldosterone of high specific activity (20 μc per μg). Plasma from pregnant subjects and from women treated with Enovid [1] was also studied. The results, in agreement with those of previous workers (7–10), indicate that the binding of aldosterone to a protein other than albumin (henceforward termed relative binding) is much less than in the case of cortisol. They provide evidence, however, that there is significant binding to such a protein or proteins in both normal and pregnant subjects at both 37° and 4° C, and that this relative binding is considerably increased by treatment with Enovid.

MATERIALS AND METHODS

Subjects. The patients treated with Enovid were chronic female schizophrenics in an open ward at the Worcester State Hospital. Each received 10 mg per day of the oral medication for 24 days, followed by 6 days without treatment. Each patient had completed at least two such cycles before being used in this study. Blood samples were taken between Days 18 and 22 of treatment. Some of the control subjects were also inmates of this ward while others were normal volunteers. No differences in plasma-binding capacity for cortisol, or in any other factors applicable to this study were observed between the volunteers and the control inmates. Blood was drawn from the controls between Days 18 and 22 of the menstrual cycle. Plasma from women in the third trimester of pregnancy was kindly made available by Dr. Brian Little of Boston City Hospital. All blood samples were drawn into heparinized tubes and the plasma separated immediately by centrifugation. When necessary, plasma was stored at −10° C before use. No change in binding capacity for either cortisol or aldosterone was observed after storage for up to 3 weeks. Penicillin G (10,000 units) (Eli Lilly) was added to all plasmas. In agreement with Slaunwhite and Sandberg (3), this did not affect binding values.

Since most of the dialyses were carried out at 37° C for 22 hours the effects of penicillin-resistant bacterial contamination are an important consideration. However, the concentrations of radioactivity followed a smooth curve

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[1] A mixture of 98 per cent norethynodrel, 17α-ethynyl-17β-hydroxy-5(10)-estrene-3-one and 2 per cent of the 3-methyl ether of 17α-ethynyl-estra-1:3:5(10)-triene-3, 17β-diol.

with time and reached a plateau from 18 to 22 hours. It seems unlikely that the dialytic process and bacterial breakdown of the complex would be in exact equilibrium during this time, although the decrease in concentration in the inner chamber after 22 hours may be due to bacterial action. There was also no evidence of conversion of steroids during the dialysis, and the results for cortisol binding agree with those determined by ultrafiltration methods (7, 8) which can be carried out in a shorter time.

Measurement of binding or relative binding. Three ml of plasma or 1 ml of plasma diluted to 3 ml with 0.9 per cent saline, together with two Teflon-coated ball bearings, was placed in the inner chamber of a dialysis cell as described by Kalant (11). A Cellophane membrane was tied over the inner portion of the cell with cotton thread. The Cellophane was stored in distilled water and a new membrane was used for each determination. Twenty ml of 0.9 per cent saline or of a solution of human serum albumin in saline was placed in the outside chamber, and radioactive or inert steroids dissolved in 1.0 ml of saline were added to this chamber. The 7-H^3-aldosterone (20 μc per μg) or 7-H^3-cortisol (28 μc per μg) used was prepared and tested for radiochemical purity as previously described for 16-H^3 steroids (12). H^3-estrone was donated by Dr. Marcel Gut; this steroid was purified by partition chromatography on Celite and tested by paper chromatography. All isotopic steroids were at least 98 per cent pure according to these criteria. The albumin used routinely was supplied by the Commonwealth of Massachusetts, Department of Public Health, Biologic Laboratories [2] in bottles containing 25 g in 100 ml solution, stabilized with 0.02 M sodium caprylate and 0.02 M sodium acetyltryptophanate. This albumin was 98 per cent pure as judged by paper electrophoresis. Before use the albumin solution was placed in a Cellophane bag and dialyzed on a mechanical shaker for 24 hours against a continuously replenished solution of physiological saline. This procedure removes a Porter-Silber chromogen which was present in the albumin. A few experiments (Table IV) were carried out with albumin that was 100 per cent pure electrophoretically. The difference in the results for the 100 and 98 per cent pure albumin were not great enough to affect the interpretation of the results, although they should be noted. In some cases the albumin content of individual plasmas was determined by the color produced with 2-(4′-hydroxybenzeneazo) benzoic acid (13), pure human serum albumin serving as standard.

Dialysis was performed for 22 hours at 37° or for 36 hours at 4° C, the cell being continuously agitated on a mechanical shaker. These times were chosen because preliminary experiments with normal plasma showed that the percentage of aldosterone (and of cortisol) bound at 37° C reached a constant maximum between 18 and 22 hours. After this the amount bound decreased, probably due to breakdown of the complex. At 4° C the rise to maximal binding was obtained after 36 hours. The percentage bound then remained constant for an indefinite time. This constant concentration after 36 hours at 4° C was also found when steroid was added to albumin and dialyzed against saline.

After equilibration an aliquot of 0.6 ml of the material in each of the inner and outer chambers was placed in a counting vial with 6 ml of ethanol, and 10 ml of scintillation fluid which contained 4 g diphenyloxazole and 100 mg 1,4-*bis*-2-(5-phenyloxolyl) benzene per L toluene (14) was added. The vials were counted in the Packard Tricarb liquid scintillation spectrometer for the time necessary to obtain at least 5,000 counts per vial, and the percentage binding or relative binding of the added steroid was determined from the formula:

$$\% \text{ bound} = \left(1 - \frac{C_o}{C_I}\right) 100$$

where C_I and C_o were, respectively, the counts in the 0.6-ml aliquots from the inner and outer chambers. Since aldosterone (and also cortisol) were shown by paper chromatography to be unchanged after dialysis, it was considered valid to count the aliquots directly without prior purification of the radioactive steroid. No individual quenching correction was used in counting the samples, since this was found in several preliminary experiments to be the same for all the aqueous phases used.

Errors of the method. The measurement of binding or relative binding by the ratio of the counts in equal aliquots from the inner and outer dialysis cells has the advantage that the result is independent of volume changes between the chambers except as a second-order effect, since in the above formula only concentration terms are involved. The change of volume could affect the binding estimates insofar as the protein concentration would alter. However, in certain experiments the volume changes were measured and in all cases found to be less than 10 per cent. This alteration in protein concentration would not be expected to affect the interpretation of the results, judged by the control experiments to be described later.

The method is subject to errors from the counting procedure and from any leakage that might occur around the dialysis membrane. Consistently high binding figures (> 90 per cent) in some experiments indicated that leakage was not important routinely. Consistent recoveries of 95 per cent of the radioactivity added indicated that binding of the radioactive steroid to the membrane was not an important factor. In any case, such binding would not affect the ratio of the counts in the two chambers, although it could theoretically be important if aliquots were continuously withdrawn for counting. For any given determination of binding or relative binding the coefficient of variation (σ) may be expressed as

$$\sigma = \frac{100 - \% \text{ binding}}{100} \times K$$

where K is the coefficient of variation in the determination of C_o/C_I which is assumed to be constant for the high counting samples used in this work. In several experiments where triplicate determinations were carried out on the same plasma which bound 10 per cent of the H^3-aldo-

[2] We are indebted to Mr. Lewis H. Larsen for making the albumin available and for advice on its purity.

TABLE I

Per cent relative binding of 7-H³-aldosterone to human plasma at 37° C; effect of different amounts of steroid

Inner chamber				
Plasma, *ml*		3	3	3
Outer chamber				
Albumin, %		4.3	4.3	4.3
7-H³-aldosterone, *mμg*		0.2	0.2	0.2
Inert *d*-aldosterone, *mμg*			10.0	3,000
Control Subjects	I	13	11	−9
	II	3	18	13
	III	21	21	19
Mean		12	17	8
Enovid-treated subject		38	39	31

sterone, the coefficient of variation of the mean value was estimated statistically at about 6 per cent. Thus:

$$K = \frac{100 \times 6}{90} = 6.6$$

By using this value of K, σ for other experiments may be calculated. Hence for triplicate determinations giving mean values for percentage binding of 10, 20, 30, and 50, the true result will be, respectively, 10 ± 6, 20 ± 5.3, 30 ± 4.6, and 50 ± 3.3. In the present paper all values quoted are the means of triplicate determinations, and a result of 10 per cent or greater is regarded as significant binding of the radioactive steroid for an individual plasma sample.

RESULTS

Influence of quantity of steroid on per cent binding. Table I shows the per cent relative binding of 7-H³-aldosterone in plasma after dialysis against 4.3 per cent albumin. This concentration of albumin was approximately equal to that in normal plasma as judged by colorimetric determinations on plasma by the method of Rutstein, Ingenito and Reynolds (13); 0.2, 10.2, and 3,000.2 mμg aldosterone were added to the dialysis cell together with plasmas from three normal subjects and one plasma from an Enovid-treated patient. In all cases, the difference in per cent relative binding when 0.2, 10.2, and 3,000.2 mμg of steroid were added was negligible. Also, four normal plasmas with 1.0 mμg 7-H³-aldosterone (Table II) and three with 3.0 mμg (Table III) were dialyzed against 5 per cent albumin. No difference in binding was observed and therefore 1.0 mμg or, more conveniently, 3.0 mμg of radioactive aldosterone was used routinely in order to reduce the counting time necessary for individual sam-

TABLE II

Per cent relative binding of 7-H³-aldosterone and 7-H³-cortisol to human plasma at 37° C

		Aldosterone				Cortisol
Inner chamber						
Plasma, *ml*		1	1	1	3	1
Saline, *ml*		2	2	2		2
						1
Outer chamber						
Albumin, %		1.6	1.6	5	5	1.6
7-H³-aldosterone, *mμg*		1.0	1.0	1.0	1.0	5
Inert *d*-aldosterone, *mμg*			3,000			7-H³-cortisol
Control subjects	A	17	15	6	8	74
	B	−11	7	1	23	76
	C	− 9	13	1	16	71
	D	17	13	5	14	77
	Mean	8.5	12	3	15	74
Enovid-treated patients	E	11	17	14	26	90
	F	30	29	16	39	92
	G	40	33	19	46	95
	H	42	37	32	47	95
	Mean	31	29	20	40	93
Pregnant subjects	J	0	4	−2	13	81
	K	17	13	2	15	80
	L	17	13	−16	21	87
	M	12			22	85
	N	6			15	83
	Mean	10	10	−5	17	83
Subject in early pregnancy	P	14	10		13	79

ples. Although the quantities added routinely are therefore 0.033 or 0.1 μg per 100 ml of plasma (giving much lower concentrations in the chambers after dialysis) these would still be lower than the normal plasma concentration of aldosterone or that attained in subjects on a low-salt diet (15), and the results above indicate that the per cent binding will be the same as if only negligible amounts of radioactive aldosterone had been added.

Relative binding of aldosterone in normal plasma. The results in Table II show that the binding of aldosterone to normal plasma diluted with saline and dialyzed against 1.6 per cent albumin is −9 to 17 per cent (mean 8.5) for four subjects, a result which is probably not significantly above zero. The binding of cortisol to the same plasmas under the same conditions was 71 to 77 per cent, which is of the same order as that obtained by Slaunwhite and Sandberg (3) who used a similar technique and the same temperature. Even when undiluted plasma was dialyzed against 5 per cent albumin, the per cent binding of aldosterone was much less than that of cortisol in diluted plasma. These results support the conclusions of previous workers (7–10) that the binding of aldosterone to proteins other than albumin is small compared with that of cortisol.

Altogether seven normal undiluted plasmas (Tables II and III) were dialyzed against 5 per cent albumin at 37° C and the per cent binding was 16.4 ± 2.2 (SE). No individual value was less than 8 per cent. At 4° C the per cent binding of three plasmas was 82, 86, and 75. This increase of binding at low temperature parallels that observed for the binding of cortisol to transcortin (3) and confirms the fact that aldosterone is bound to proteins other than albumin, since the binding of aldosterone to albumin is largely independent of temperature (Table IV), in agreement with the results of Daughaday and co-workers (10).

Colorimetric estimation (13) on several plasmas indicated an albumin content of 4.3 per cent for normal plasma and lower values in pregnant and Enovid-treated subjects. The method was not considered extremely accurate, however, and even though dialysis was performed against 5 per cent albumin, the question arose of whether the values obtained for aldosterone binding could have been caused by a greater concentration of albumin in the inner than the outer chamber. This possibility is illustrated by the insignificant aldosterone relative binding obtained when diluted plasma, the albumin concentration of which was about 1.3 per cent, was dialyzed against 5 per cent albumin (Table II), and by the appreciable binding of aldosterone to albumin dialyzed against saline (50 per cent, Table IV), which is of the same order as that of cortisol. It was therefore thought necessary to do other experiments to examine the possible effect of differing albumin concentrations in the two chambers. Estrone has been reported to be bound strongly to albumin and, to a negligible extent, to other proteins (3). Although we have found that when 1 to 3 mμg of estrone (20 μc per μg) is added to 3 ml of plasma, binding to other proteins can be appreciable, we have confirmed the high binding of the steroid to albumin (90 per cent, Table IV) under several conditions of dialysis. If the amount of estrone is raised to 10.0 μg per 3 ml plasma (initial concentration), the binding to albumin is unchanged (Table IV) while that to other proteins is reduced to very small amounts (Table III). At this concentration estrone can therefore be used as an indicator of albumin binding.

Table III shows results obtained with three plasmas in which the binding capacity for aldosterone and for estrone (10 μg per 3 ml) was measured by dialysis against 5 per cent albumin. The per cent binding of aldosterone at 37° C was 17, 24, and 13, and the corresponding values for estrone were −1, 8, and 4. The values for per

TABLE III

Per cent relative binding of aldosterone and estrone to plasma of control subjects at 37° and at 4° C

Inner chamber	3 ml Plasma	3 ml Plasma
Outer chamber	5% Albumin 3.0 mμg 7-H^3-aldosterone	5% Albumin 5.0 mμg 16-H^3-estrone 10 μg inert estrone
	17	−1
	24	8
37°	13	4
Mean	18	4
	82	−50
4°	86	−75
	75	−120
Mean	81	−82

TABLE IV
Per cent binding of radioactive steroids to albumin *

Inner chamber Albumin, % / Outer chamber	1.6 5 mμg 16-H^3-estrone +10 μg inert estrone in saline	5 5 mμg 16-H^3-estrone in saline	5 5 mμg 16-H^3-estrone +10μg inert estrone in saline	5 5 mμg 7-H^3-cortisol in saline	5 5 mμg 7-H^3-aldosterone in saline
37°	83	92 93†	92	48 57†	40 50†
4°	90	93 95†		52 66†	51 56†

* The first figure in each column represents % binding to the "98% pure" albumin used throughout these experiments. The second figure, marked†, represents % binding to a sample of albumin that was 100% pure as judged by paper electrophoresis.

cent binding of aldosterone at 4° C were 82, 86, and 75, and for estrone −50, −74, and −120. Negative values presumably reflect a greater concentration of albumin in the outer chamber. The increased binding of aldosterone at low temperature would be expected if the protein responsible behaved like transcortin (3), while the negative values for estrone would result from a higher concentration of albumin in the outer chamber, since it has been shown (Table IV) that the binding of estrone to the albumin used in these experiments is increased at low temperature. Because of the high binding of estrone to albumin, such increases could be clearly demonstrated only when dilute solutions of the albumin were dialyzed against saline. However, if albumin solutions of only slightly differing concentration were present in both chambers and the binding were high, then the relative binding could have a high temperature coefficient. Since the binding of aldosterone to albumin is not strongly dependent on temperature (Table IV) (10) the high values of binding found for this hormone at 4° C must be due to a protein other than albumin.

Relative binding of aldosterone in plasma of pregnant subjects. In agreement with the results of previous workers (3) the binding of cortisol was found to be elevated in plasmas from five subjects in the last trimester of pregnancy. The value for the plasma of one subject in early pregnancy was increased to a lesser extent. However, the results for aldosterone relative binding (Table II, Figure 1), although significant (17 ± 1.7), were of the same order as those of the controls (16.4 ± 2.2) in all these plasmas. Previous workers (16, 17) have reported and we have confirmed, that the concentration of albumin is lower in pregnancy plasma than in normal plasma, so that it is most unlikely that the values obtained could be due to binding by albumin. The effect of this lowered albumin concentration in the inner chamber would be to lower the binding found in our experiments, and it is therefore not possible to conclude from the result that there is no increase of aldosterone binding in pregnancy, but any such increase is probably very small.

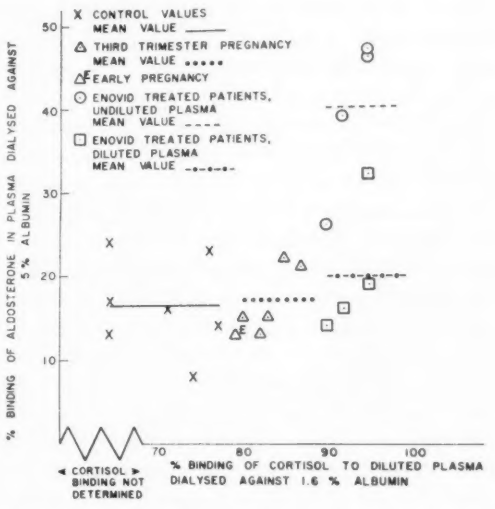
X CONTROL VALUES MEAN VALUE
△ THIRD TRIMESTER PREGNANCY MEAN VALUE
△E EARLY PREGNANCY
○ ENOVID TREATED PATIENTS, UNDILUTED PLASMA MEAN VALUE
□ ENOVID TREATED PATIENTS, DILUTED PLASMA MEAN VALUE
% BINDING OF ALDOSTERONE IN PLASMA DIALYSED AGAINST 5 % ALBUMIN
50
40
30
20
10
70
80
90
100
◄ CORTISOL ► BINDING NOT DETERMINED
% BINDING OF CORTISOL TO DILUTED PLASMA DIALYSED AGAINST 1.6 % ALBUMIN

FIG. 1. THE RELATIONSHIP BETWEEN THE RELATIVE BINDING OF ALDOSTERONE IN UNDILUTED PLASMA (ON THE ORDINATE) TO THAT OF CORTISOL IN PLASMA DILUTED 1:3 (ON THE ABSCISSAE) FROM NORMAL, PREGNANT AND ENOVID-TREATED WOMEN. All dialyses were performed with equal concentrations of albumin in the inner and outer chambers of the dialysis cell, except for points marked ⊡. These points show the relationship between the binding of aldosterone in 1:3 diluted plasma from Enovid-treated women, dialyzed against three times its concentration of albumin, to the binding of cortisol in the same plasma dialyzed against an equal concentration of albumin.

Relative binding of aldosterone to plasma of patients treated with Enovid. In Enovid-treated patients the capacity of the plasma to bind cortisol is increased even more than in pregnancy, while the aldosterone relative binding in these patients was increased above normal under all conditions of dialysis (Table II, Figure 1). When diluted plasma was dialyzed against 1.6 per cent albumin the per cent binding was 31 ± 7 (SE). In undiluted plasma dialyzed against 5 per cent albumin the value was 40 ± 5 as compared with values of 16.4 ± 2.2 and 17 ± 1.7 in normal and pregnancy plasmas, respectively.

The albumin concentrations in the plasma of Enovid-treated patients, while not so low as those in pregnancy plasma, were lower than those in normal plasma, so that the increased binding cannot be due to larger albumin concentration in the inner chamber. This is further shown by the per cent binding of 20 ± 4 in diluted plasma (containing about 1.5 per cent albumin) dialyzed against 5 per cent albumin (Table II). The binding of aldosterone at 4° C in undiluted plasma dialyzed against physiological saline was over 90 per cent, as shown in Table V.

Table V also shows the per cent binding of aldosterone to diluted and undiluted plasma from Enovid-treated subjects after dialysis against 5 per cent albumin and against saline in both cases. An excellent correlation was found between the per cent binding of cortisol to diluted plasma (dialyzed against 1.6 per cent albumin) and aldosterone to undiluted plasma (dialyzed against 5 per cent albumin) in normal, pregnant, and Enovid-treated subjects (Figure 1). This correlation remained good even when the results for aldosterone relative binding in diluted plasma (containing about 1.6 per cent albumin) dialyzed against 5 per cent albumin were plotted against the results for cortisol binding in diluted plasma dialyzed against 1.6 per cent albumin. These results are shown in Figure 1 for Enovid-treated patients.

In confirmation of the results of Sandberg and co-workers (9), obtained at 4° C, it was found that 1.0 μg of aldosterone did not appreciably reduce the binding of cortisol in a normal plasma at 37° C (76 per cent), while 2, 5, and 10 μg of aldosterone reduced this binding to 68, 56, and 48 per cent, respectively. Similar amounts of cortisol consistently produced much greater reduction in the percentage binding of 7-H^3-cortisol to normal plasma at 37° C. However, the binding of aldosterone to the plasma of an Enovid-treated patient (38 per cent) was not affected by the addition of 2 μg of cortisol but was reduced to 30, 19, and 15 per cent when 5, 10, and 20 μg of cortisol, respectively, were added to the plasma.

TABLE V

Per cent binding of H^3-aldosterone to plasma of Enovid-treated patients at 36° and at 4° C; dialysis against albumin and against saline

Inner chamber				
Plasma, *ml*	1	1	3	3
Saline, *ml*	2	2		
Outer chamber	5% Albumin 1 mμg H^3-aldosterone	Saline 1 mμg H^3-aldosterone	5% Albumin 3 mμg H^3-aldosterone	Saline 3 mμg H^3-aldosterone
37°	30	48	44	70
4°	92	93	95	98

DISCUSSION

The above results provide strong evidence that there is a significant binding of aldosterone to a protein or proteins other than albumin in human plasma. The results are in agreement, qualitatively at least, with those of Sandberg and colleagues (9) and the Daughaday group (10). The latter found no binding of aldosterone to proteins other than albumin in normal, pregnant or estrogen-treated subjects at 37° C, although they reported a binding at 4° C. In the present experiments we found a definite binding of aldosterone to plasma proteins other than albumin in control and pregnant subjects at 37° C. This binding was increased by treatment with Enovid, and in control plasma the binding of aldosterone was very much increased by lowering the temperature to 4° C. The slight discrepancy between our results and those of Daughaday and co-workers could be explained by differences in the sensitivity of the methods used. In our studies the binding of aldosterone in normal subjects at 37° C, while significant, was small, and it is possible that the method of the Daughaday group did not detect this binding; their result, a lowering of aldosterone binding at 37° C from 63 to 58 per cent on addition of cortisol, also indicates this possibility.

The nature of the proteins binding aldosterone has not been defined in these studies. In normal

plasma the capacity of the protein for steroid is much higher than is the normal plasma concentration of aldosterone (1 mμg per 3 ml plasma) since 3,000 mμg of aldosterone had to be added to 3 ml of plasma to produce an appreciable lowering of the percentage binding, and even this is probably not a significant effect. This concentration (minimum after equilibrium, 1 μg per ml) approaches the amount of steroid needed to lower the binding of cortisol under similar conditions and suggests that the protein binding aldosterone may be transcortin. This conclusion is supported by the results in Enovid-treated patients in whom both cortisol and aldosterone binding was increased, and a good correlation was observed between these increases. The reduction of cortisol binding by aldosterone (although not so great as that produced by a similar amount of cortisol) is further evidence that aldosterone and cortisol are bound to the same protein, which has a greater affinity for cortisol than for aldosterone. In pregnancy, however, cortisol binding increases while that of aldosterone does not, suggesting that the protein binding aldosterone is not necessarily transcortin. Sandberg and colleagues (9) have shown in normal plasma at 4° C that the binding of aldosterone is rapidly reduced by the addition of cortisol, but we have found that the addition of cortisol, in amounts similar to that present in pregnancy plasma, has no effect on the binding of aldosterone at 37° C in Enovid-treated patients. It seems unlikely, therefore, that the high plasma cortisol levels present in pregnancy account for the failure to demonstrate increased binding of aldosterone in pregnancy plasma. It is possible that the presence of steroids other than cortisol may affect the binding of aldosterone, but protein fractionation studies on the plasma will be necessary before further conclusions can be drawn.

The physiological significance of the present *in vitro* results can only be clarified by the investigation in pregnant and Enovid-treated patients of *in vivo* phenomena that might be correlated with changes in plasma binding of aldosterone, such as the volumes of distribution and turnover rate of the hormone. Methods for the determination of these values have recently been devised, and it has been found that in normal subjects injected aldosterone spreads rapidly into an initial volume of 25 L and more slowly to 40 L (18). These volumes of distribution are much larger than those reported for cortisol (19), and the turnover rate of aldosterone is much greater than that of cortisol (18). One reason for this difference in the distribution and turnover rates of the steroids could be the much stronger binding of cortisol to plasma proteins. A situation analogous to that between cortisol and aldosterone has been noted in studies of the binding and transport of triiodothyronine and thyroxine (20). An increased binding of aldosterone in plasma would therefore be expected to decrease the volume of distribution and the turnover rate of the steroid. From the results of the present study no alteration in these values would be expected in pregnant as compared with normal subjects. Jones and co-workers (21) found no alteration in the urinary excretion of the free aldosterone expressed as a percentage of the secretion rate during pregnancy and, assuming this parameter to reflect the ratio of the blood concentration of the hormone to the secretion rate, they suggested that the turnover rate of aldosterone is probably the same in pregnant and normal subjects. Actual determination of the turnover rate and volume of distribution of injected 7-H^3-aldosterone in four women in the third trimester of pregnancy has shown no significant change in these values as compared with normal subjects (22). In the case of Enovid-treated subjects a decrease in the turnover rate would be expected from the increased binding found in the present experiments. This has not as yet been investigated.

The mechanism of action of Enovid in increasing the binding of aldosterone and cortisol in plasma will be the object of further studies. It is not known whether this activity is correlated with its estrogenic or progestogenic potency. The major point of the present studies was to demonstrate for the first time that under certain conditions the binding of aldosterone could be increased, and that this effect was correlated with increases in transcortin activity.

SUMMARY

The binding of 7-H^3-aldosterone to proteins in human plasma has been investigated by a microdialysis technique. The percentage relative binding of physiological amounts of aldosterone to

undiluted plasma of normal women after dialysis against 5 per cent human serum albumin was 16.4 ± 2.2 (SE). Corresponding values for late pregnancy plasma were 17 ± 1.7, and for women treated with Enovid the percentage relative binding was 40 ± 5. These values are much smaller than those obtained for the percentage binding of cortisol to plasmas obtained from the same subjects.

It has also been found that aldosterone and cortisol are bound to albumin to about the same extent. However, dialysis of 7-H^3-aldosterone at 37° and at 4° C against different concentrations of albumin provided evidence that the binding obtained in plasma dialyzed against albumin was not due to differences in the concentration of albumin in the plasma and in the outer dialysis chamber. Similar experiments with labeled estrone confirmed this conclusion.

The results are therefore interpreted as strong evidence that there is significant binding of aldosterone in human plasma to a protein or proteins other than albumin. Any increase of this binding during pregnancy is probably small, but there is a marked increase in relative binding after treatment with Enovid.

ADDENDUM

In five subjects treated with 0.3 mg per day of ethinylestradiol 3-methyl ether the mean percentage binding of cortisol and of aldosterone was 89 and 38 per cent, respectively. These values resemble those obtained with a dose of 10 mg per day of Enovid. Three patients on 0.1 mg per day of ethinylestradiol 3-methyl ether showed no increase in relative binding of aldosterone. These results indicate that the increased binding of aldosterone caused by Enovid is probably due to its estrogenic potency. The authors incline to the conclusion that the effective estrogen levels in the pregnant subjects did not reach the threshold needed to raise the plasma binding of aldosterone.

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INSULIN-RESISTANT DIABETES ASSOCIATED WITH INCREASED ENDOGENOUS PLASMA INSULIN FOLLOWED BY COMPLETE REMISSION

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Insulin unresponsiveness may be associated with a multitude of conditions and reflect diverse etiologies (1). In most cases of chronic insulin resistance it has been possible to demonstrate insulin antagonists in the plasma which have many of the characteristics of antibodies (2–4). The resistance to insulin has usually been attributed to these antibodies, but several cases of insulin resistance associated with increased amounts of insulin in the plasma have been reported (5–8). In these cases tissue unresponsiveness was postulated as the cause for the resistance. In all of these cases circulating insulin could be demonstrated only at a time when the patients were receiving large doses of exogenous insulin, and therefore the insulin in the plasma probably represented injected insulin. We recently have had occasion to study a patient with severe insulin resistance whose plasma contained large amounts of insulin 3 months after her last known injection. Over the ensuing 18 months she had a gradual but complete remission of both diabetes and insulin unresponsiveness. The purpose of this paper is to report studies on this case which indicate that the insulin-like activity in her plasma did, in fact, represent endogenous insulin and that the cause for her insulin resistance was probably due to decreased tissue responsiveness to the hormone. The explanation for her remission is unknown, although there are occasional reports in the literature of spontaneous remission in severe diabetics (9). As far as we know this is the first reported case of insulin-resistant diabetes associated with greatly increased levels of endogenous insulin.

METHODS

The rat hemidiaphragm studies were done as follows. Fed rats weighing between 120 and 170 g were killed by a blow on the head; the diaphragms were removed as rapidly and with as little trauma as possible. Each hemidiaphragm was trimmed, blotted, weighed on a torsion balance and then put into a flask holding 2 ml of incubation medium which contained 0.5 ml of 1,000 mg per 100 ml glucose in Krebs-Ringer bicarbonate buffer (pH 7.4), variable amounts up to 1 ml of dialyzed plasma to be tested, and enough Krebs-Ringer bicarbonate buffer to make the final volume 2 ml. When standard insulin solutions were tested, appropriate dilutions of a stock insulin solution (40 U per ml) were made with Krebs-Ringer bicarbonate buffer. Control flasks with the appropriate amount of plasma or buffer but without tissue were included in each experiment for the determination of the initial glucose concentration. The flasks were gassed with 95 per cent O_2 and 5 per cent CO_2 and incubated for 90 minutes at 37° C on a Dubnoff metabolic shaker. Glucose concentrations were measured on duplicate 0.5 ml aliquots of the incubation medium with glucose oxidase (Glucostat, Worthington Biochemical Corp.). The difference between the initial and final glucose concentration was calculated and the results expressed as milligram of glucose uptake per gram of muscle. In each experiment the test samples of plasma and insulin standards were assayed in triplicate. Glycogen deposition in the rat diaphragm at the end of the incubation period was measured by the anthrone method (10).

Protein fractionation of plasma was done by starch block electrophoresis and veronal buffer (pH 8.6), ionic strength 0.05 (11). After electrophoresis the block was cut into 1 cm segments and the protein recovered by centrifugation. Protein concentration was determined either by ultraviolet absorption at 284 mμ in the Beckman DU spectrophotometer or by the modified method of Folin (12). Protein solutions of two adjacent segments were combined, dialyzed against distilled water and then lyophilized. The resulting protein dissolved in Krebs-Ringer bicarbonate buffer was assayed in the rat hemidiaphragm system for insulin-like activity. Equal volumes of the various fractions were tested. In one experiment 20 μU of insulin-I^{131} obtained from Abbott Laboratories was added to 1 ml of plasma prior to electrophoresis. In this experiment the plasma fractions were also assayed for radioactivity in a well-type scintillation counter and counted to an accuracy of 5 per cent.

The rate of disappearance of insulin-I^{131} was measured as follows: 40 μc of insulin-I^{131} (Abbott Labs.), representing 0.25 U of insulin, was rapidly injected intravenously. Blood samples were obtained just before and at 2.5, 15, 30, 60, 120, 180, and 240 minutes and 24, 48,

and 72 hours after the injection. Thyroid utilization of iodide was blocked with methimazole (Tapazole). The trichloroacetic acid (TCA)-precipitable radioactivity was determined according to the method of Mortimore, Tietze and Stetten (13). Since no attempt was made to distinguish denatured TCA-precipitable insulin-I^{131} from undamaged insulin-I^{131}, the latter part of the disappearance curve may be falsely prolonged (14).

Adipose tissue from the buttock was obtained by biopsy under local anesthesia. The tissue was immediately sliced with a Stadie-Riggs microtome, blotted, weighed and then each slice put into a flask containing 2 ml of Krebs-Ringer bicarbonate buffer and 250 mg per 100 ml glucose with or without insulin. When insulin was present its final concentration was 0.1, 0.5, or 1 U per ml. In the usual experiment, incubations were done in quadruplicate. Chilling was avoided and the incubation conditions were similar to those for rat diaphragms. The glucose uptake per gram of tissue was calculated as for the rat diaphragm studies. It is realized that these amounts of insulin are large and much greater than those detected by rat epididymal fat pad (15). In preliminary experiments with normal human adipose slices it was found that 0.1 U per ml was the smallest insulin concentration giving a consistent effect. The trauma to the tissue during the biopsy and subsequent slicing may be responsible for this decreased sensitivity. The effect of insulin on glucose uptake by leukocytes was measured by the procedure of Weinberg (16). The amount of insulin, 0.6 U per ml, used in these studies was not physiologic but was necessary for the demonstration of an insulin effect. The study of the *in vivo* oxidation of glucose-1-C^{14} to $C^{14}O_2$ was kindly performed by Dr. Stanton Segal of this Institute (17).

Insulin antibody plasma was obtained from a patient with chronic insulin resistance; 0.1 ml of this plasma was capable of abolishing the effect of 2×10^{-3} U of insulin in the rat diaphragm test system (18). Intravenous insulin tolerance tests were performed with glucagon-free insulin kindly supplied by Eli Lilly and Co.

CASE REPORT [1]

This 18 year old Negro female had enjoyed excellent health until age 15 when she noted the onset of tiredness, weight loss, polyuria, polydipsia and polyphagia. A diagnosis of diabetes mellitus was made on the basis of hyperglycemia and glycosuria and she was admitted to the West Jersey Hospital in Camden, N. J. She was placed on a diabetic diet and insulin, but despite increasing the dosage to 50 U per day she continued to have constant glycosuria and acetonuria, although her symptoms diminished. She was discharged from the hospital, taking 50 U NPH insulin each morning and a diabetic diet. Because of persistent hyperglycemia, glycosuria and acetonuria, her insulin dosage was gradually increased over the next 6 months until she was receiving 600 U per day in divided doses. During this period she was asymptomatic, had regained some weight and had never experienced any insulin reactions. She was then referred to the diabetic clinic of the University of Pennsylvania Hospital. Over the next 3 months the insulin dosage was increased further without any apparent change in her glycosuria. Fasting blood sugars were frequently over 300 mg per 100 ml. She was admitted to the University of Pennsylvania Hospital in October 1958 for evaluation. Her system review was negative except for increasing pigmentation of the skin without light sensitivity, of 1 year's duration, and amenorrhea for the same period.

The past history and family history were unremarkable except that her paternal grandmother had diabetes adequately controlled with tolbutamide. Physical examination was normal except for acanthosis nigricans (confirmed by skin biopsy), carotenemia and small, tender subcutaneous lumps in the thighs at the sites of insulin injections. Initial laboratory data included: hemoglobin 16.1 g; white count 3,900 cells per mm^3 with 46 per cent neutrophils, 2 per cent eosinophils, 4 per cent monocytes and 48 per cent lymphocytes; blood-urea-nitrogen 8 mg per 100 ml; fasting blood sugar 186 mg per 100 ml and 2 hour postprandial blood sugar 351; serum proteins 6.8 g with 3.1 g albumin and 3.7 g globulin; cholesterol 177 mg per 100 ml; negative urinalysis except for 4+ glycosuria and acetonuria; normal electrolytes and CO_2 values; serum carotene 107 μg per 100 ml.

Attempts were made to control the diabetes by increasing her daily insulin dosage but without much success. She continued to have constant glycosuria and acetonuria. Once her urine was negative for sugar and her blood sugar was 90 mg per 100 ml after an 18 hour fast and 6,000 U insulin. Cortisol therapy was not beneficial, and while on steroids, her insulin dosage was increased to 14,000 U of regular insulin per day. Her 24-hour urinary excretion of glucose was 292 g. Steroids were discontinued and metahexanamide (Metahexamide) therapy (300 mg per day) was instituted. She continued to have hyperglycemia on this therapy and insulin was increased to 38,000 U per day. Despite this amount of insulin her 24 hour urinary glucose output ranged from 109 to 190 g. At this point insulin and metahexanamide therapy was discontinued and she was treated with 200 mg phenethylbiguanide (DBI) daily. Urinary glucose content increased to approximately 245 g per 24 hours, and the addition of insulin had no effect. At no time during her hospitalization was there any evidence of hypoglycemia or definite acidosis, although on one occasion a CO_2 of 16 mEq per L and a positive undiluted plasma acetone test was obtained. These quickly returned to normal without any special therapy.

Immunologic studies indicated a negative skin test to Lente insulin, regular beef insulin and pork insulin. Although she had reported tender lumps at the site of insulin injections, there had never been any generalized allergic manifestations. Antibodies to insulin could not be demonstrated by passive transfer. Intravenous insulin

[1] We are indebted to Drs. Bruce Carruthers and Stanley Spoont, University of Pennsylvania Hospital, for referral of this patient and the summary of her hospitalization at that hospital.

injections of 100 U of either beef or pork insulin caused no significant decrease in the blood sugar.

Since her hyperglycemia, glycosuria and acetonuria were unaffected by insulin, she was treated by diet alone and 1 month later was transferred to the Clinical Center, National Institutes of Health, for further studies. She felt well except for some dryness of the mouth and excessive hunger. Her physical examination showed no change when compared with that of her admission to the University of Pennsylvania Hospital. Her initial laboratory workup included a normal urinalysis except for a 2+ urine sugar and positive acetonuria; hematocrit 43 per cent; WBC 2,900 per mm^3 with 40 per cent mature polymorphonuclear leukocytes, 50 per cent lymphocytes, 9 per cent monocytes and 1 per cent eosinophils. Sedimentation rate was 15 mm per hour; fasting blood sugar 252 mg per 100 ml; total cholesterol 200 mg per 100 ml; CO_2 26 mEq per L; serum sodium, chloride and potassium 141, 104, and 4.5 mEq per L, respectively; unesterified fatty acids 1.47 mEq per L; total protein 6.7 g with 3.3 g albumin and 3.4 g globulin; serum electrophoresis indicated albumin 2.86 g, α_1-globulin 0.33 g, α_2-globulin 0.52 g, β-globulin 0.98 g and γ-globulin 1.82 g; transaminase 30; alkaline phosphatase 1.8 Bodansky units; 2 per cent retention of sulfobromophthalein at the end of 45 minutes; cephalin flocculation 1+ in 24 hours and 2+ in 48 hours; basal metabolic rate −8 per cent; protein-bound iodine 5.0 μg per 100 ml; plasma cortisol 16 μg per 100 ml; 24-hour urinary 17-hydroxycorticosteroids and 17-ketosteroids 4.5 and 3.5 mg, respectively.

She was asymptomatic without insulin therapy despite 24-hour urine glucose excretion of up to 332 g while taking a 3,175 caloric diet containing 300 g carbohydrate. When allowed to eat *ad libitum* she consumed 6,000 calories per day and her urinary glucose excretion rose to approximately 600 g per 24 hours. Acetonuria was present constantly and 24-hour urine samples contained as much as 20 g of ketone bodies. Fasting blood sugars ranged from 155 to 285 mg per 100 ml and postprandial sugars were as high as 519. Despite this glycosuria and acetonuria her weight actually increased from 42.3 kg at the time of admission to 45.6 kg at the time the initial studies, which serve as the basis for this report, were undertaken.

Two months after her admission she developed mild indigestion, heartburn, anorexia, increased thirst, nocturia and nausea, and she lost 1.1 kg of weight over a 2-day period. Her fasting blood sugar was found to be 420 mg per 100 ml and her CO_2 17 mEq per L. Her plasma acetone was positive in a 1:3 dilution whereas previously it had been negative. Sodium was 128, chloride 100, and potassium 5.1 mEq per L. Physical condition was unchanged except for increased respiratory rate later accompanied by typical Kussmaul respiration and a tender liver edge on deep inspiration. Dehydration was not striking. Since she had not received insulin for over 3 months, treatment was started with amounts of insulin that would be considered small in comparison with her previously tolerated doses. Over the next several hours she received 300 U regular insulin i.v. and fluid therapy consisting of 0.17 M lactate and 0.5 N saline plus 40 mEq KCl. She ate a regular diet. Several hours later her blood sugar had risen to 590 mg per 100 ml and CO_2 was 16 mEq per L with no change in her clinical status. She was given another 825 U regular insulin i.v. over the ensuing 4 hours with a resultant fall in blood sugar to 300 mg per 100 ml but her CO_2 had also declined to 14 mEq per L. Because of the continuing decline in CO_2 she was given an injection of 1,000 U regular insulin i.v. and fructose was added to the intravenous fluid therapy. Fifteen minutes later she developed fever to 104°F, shaking chills, and generalized urticaria. The various intravenous fluids being administered at the time were tested and were pyrogen-free. Since this might be an allergic reaction to insulin, she was given 100 mg cortisol hemisuccinate i.v. and 1,000 U regular insulin i.m. every hour for the next 4 hours. At the end of this time her blood sugar was 450 mg per 100 ml and CO_2 had decreased to 12 mEq per L. Fever and chills did not recur and urticaria disappeared. In view of the further deterioration of the blood sugar and CO_2, she was given 12,000 U of regular insulin in saline i.v. over the next 6 hours. At the end of this she felt better and her CO_2 was found to be 15 mEq per L and blood sugar 320 mg per 100 ml. During the next 12 hours she continued to receive intravenous fructose, lactate and saline as well as 20 mg prednisone and 3,000 U insulin. She was then completely asymptomatic, with a blood sugar of 207 mg per 100 ml and a CO_2 of 24 mEq per L.

For the next 2 days she received 1,000 U regular insulin i.m. per day and 40 mg prednisone. Intravenous fluids were discontinued and she consumed her usual diet. Although she again had hyperglycemia, her CO_2 was normal. The next day she again had anorexia and nausea, and CO_2 was 14 mEq per L, with a blood sugar of 365 mg per 100 ml. Plasma acetone was positive in a dilution of 1:8 whereas it had been negative the previous day. An insulin-I^{131} half-time disappearance of 2 hours had been found the previous day as compared with one of 10 hours 1 week before. Because of this change in insulin disappearance rate and because the patient was still on steroids, insulin therapy was begun with small doses. She was given 25 U regular insulin i.v.; 30 minutes later her blood sugar was 375 mg per 100 ml as compared with 365 before the insulin. Intravenous lactate, fructose and saline were again administered as well as an infusion of 1,000 U insulin per hour. Later in the day there was some clinical improvement and CO_2 had risen to 17 mEq per L although her blood sugar was 420 mg per 100 ml. Plasma acetone was still positive in a dilution of 1:4. She was greatly improved clinically the next morning: CO_2 was 23 mEq per L and blood sugar was 115 mg per 100 ml.

Because of the recurrent acidosis it was decided to decrease the prednisone and attempt to maintain her on 2,000 U insulin per day. Several days later CO_2 was 14 mEq per L and fasting blood sugar 183 mg per 100 ml.

TABLE I

Blood glucose response after intravenous insulin

Date	Insulin	Blood glucose minutes 0	15	30	45	60	90
	U	*mg per 100 ml*					
Sept. 1959	10	150	145	140		124	114
Sept. 1959	200	186	141	139		135	120
Sept. 1960	6	54	17	22	31	32	46

Since she was asymptomatic and it was not clear whether fluid therapy or massive doses of insulin were responsible for her improvement during her two previous episodes of acidosis, it was decided to try i.v. fructose and saline without insulin. Although there was a slight decrease in the plasma free fatty acids [2] during the infusion, the blood sugar rose to 519 mg per 100 ml. The next morning her fasting blood sugar was 267 mg per 100 ml and CO_2 15 mEq per L. Intravenuos 0.17 M lactate therapy, again without insulin, was administered. This therapy produced no change in plasma free fatty acids, although there was a slight fall in the blood sugar. Over the next 4 days CO_2 gradually rose to 28 mEq per L despite the fact that she was given no insulin therapy nor further intravenous fluid. The massive doses of insulin given during her first two episodes of acidosis were of questionable benefit. No apparent cause for these three episodes of acidosis could be found, and large amounts of insulin were present in her plasma just before insulin treatment of the first episode was initiated. She had no further episodes of acidosis and remained asymptomatic with no insulin therapy. She continued to have constant glycosuria and acetonuria. At time of discharge, in May 1959, she weighed 49.7 kg and was advised to take no insulin but to follow a 2,100 calorie diabetic diet ($C_{200}P_{100}F_{100}$).

She was readmitted to the Clinical Center 2 months later for subcutaneous fat biopsy to assess the insulin responsiveness of this tissue. While at home she was asymptomatic and her glycosuria, although still present, was diminished. Her weight on admission was 55.5 kg and the physical examination showed no change from her previous admission. Fasting blood sugars ranged from 150 to 244 mg per 100 ml and 4 p.m. blood sugars as high as 700 mg per 100 ml were recorded. Table I summarizes the response of her blood sugar to various amounts of glucagon-free insulin given intravenously during this admission and 1 year later when both the diabetes and insulin resistance were in complete remission. The results in September 1959 confirmed the fact that she was still insulin-resistant at that time. Whereas during her previous admission she had excreted up to 332 g of glucose per 24 hours on a 3,125 calorie diet, on this admission her 24-hour glucose excretion never exceeded 150 g and acetonuria was absent. A subcutaneous fat biopsy was obtained and, after an uneventful postoperative course, she was again discharged on her previous diet.

Soon after discharge the patient noted a further decrease in glycosuria and in November 1959 her urine tests became negative for sugar. Concomitantly, menstruation reappeared and the severity of the acanthosis nigricans decreased. Blood sugars, both fasting and postprandial, were found to be within normal limits. An oral glucose tolerance test done at the University of Pennsylvania Hospital revealed normal carbohydrate tolerance with a tendency toward hypoglycemia during the third to fifth hour of the test. Her response to a cortisone glucose tolerance test (19) was also normal and she was allowed an unrestricted diet. Over the following 10 months she gained 7 kg and was asymptomatic. She was readmitted to the Clinical Center in August 1960 for re-evaluation. Physical examination showed that she was entirely normal except for the acanthosis nigricans, which had cleared considerably. Her weight was 63 kg. Urinalysis was normal and glycosuria was never observed. Fasting blood sugar was 60, blood-urea-nitrogen 12, and cholesterol 167 mg per 100 ml; total protein 6.7 g per 100 ml with 3 g albumin; protein-bound iodine 5.8 μg per 100 ml; 17-ketosteroids 4.6, and 17-hydroxycorticosteroids 5.2 mg per 24 hours. An oral glucose tolerance test (100 g glucose) gave the following results: fasting 60, 1 hour 73, 2 hours 54, 3 hours 67, 4 hours 42, and 5 hours 60 mg per 100 ml. At no time during the test were there any symptoms indicative of hypoglycemia. The results of an intravenous insulin tolerance test (0.1 U per 1 g) are summarized in Table I. In contrast to the results in September 1959, 6 U of insulin now produced severe hypoglycemia, indicating a normal insulin responsiveness. Previously, 200 U i.v. had produced only a moderate fall in blood glucose levels. The half-time disappearance of a tracer dose of insulin-I^{131} given i.v. was 30 minutes, which is normal for non-insulin treated subjects (14). This rate is again markedly different from what it was in February 1959 during the peak of her insulin unresponsiveness (Figure 6). During this admission she was given 1 g sodium tolbutamide i.v. over a 30-minute period with the following results: blood sugar before infusion 62 mg per 100 ml, at end of infusion 29, 30 minutes later 43, 60 minutes later 51, and 90 minutes later 55 mg per 100 ml. She was discharged from the hospital on an unrestricted diet.

RESULTS

Plasma obtained 3 months after the patient's last known injection of insulin, when she was still diabetic, greatly stimulated glucose uptake by the isolated rat hemidiaphragm, as shown in Figure 1. Although 1 ml of normal plasma also increased glucose uptake by diaphragm, the effect was small in comparison. On the basis of the insulin stand-

[2] We are indebted to Dr. Peter Mueller, National Cancer Institute, who kindly performed these determinations.

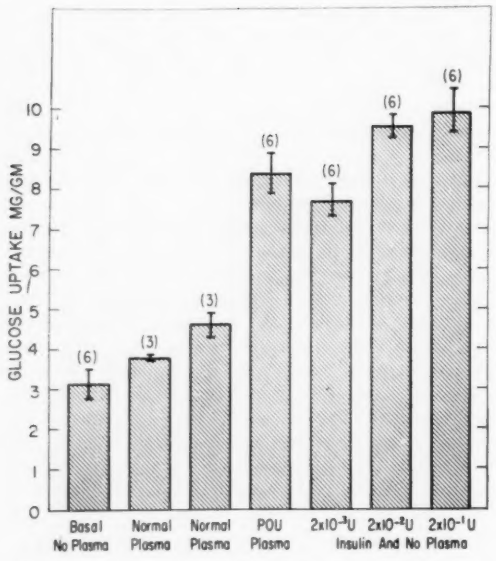

FIG. 1. EFFECT OF PLASMA ON GLUCOSE UPTAKE BY ISOLATED RAT DIAPHRAGM.

ards used, her plasma contained at least 2 mU of insulin per ml. In this test system 20 mU produced a maximal stimulation. In addition to augmenting glucose uptake, the patient's plasma stim-

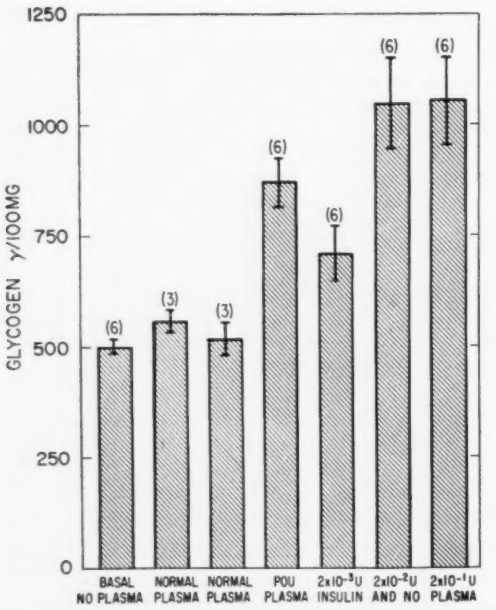

FIG. 2. EFFECT OF PLASMA ON THE GLYCOGEN DEPOSITION BY ISOLATED RAT DIAPHRAGM.

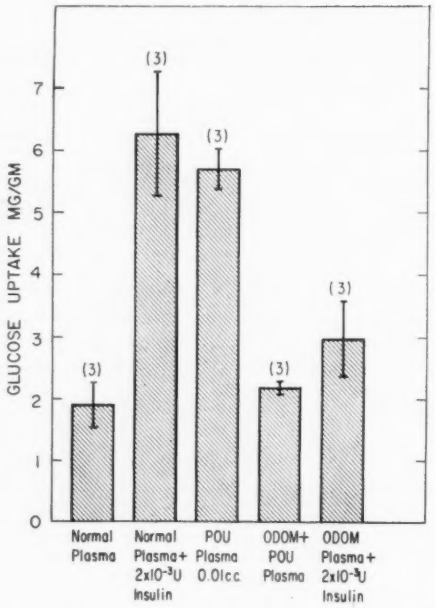

FIG. 3. INHIBITION OF INSULIN EFFECT OF POU PLASMA BY INSULIN ANTAGONIST PLASMA.

ulated glycogen synthesis in the rat diaphragm while normal plasma did not differ from buffer alone (Figure 2). Again, in comparison with the insulin standards, the patient's plasma contained between 2 and 20 mU of insulin per ml. When the plasma was assayed by a specific immunological method it contained between 17 and 27 mU of insulin per ml.[3]

Figure 3 indicates that the glucose uptake stimulatory effect of the patient's plasma was abolished by the addition of plasma containing a circulating insulin antagonist. The addition of 2 mU insulin to 1 ml normal plasma increased the glucose uptake from 1.88 to 6.24 mg per g. In the presence of 0.01 ml of the patient's plasma, the glucose uptake was 5.70 mg per g. The addition of 1 ml of antagonist plasma to 0.01 ml of the patient's plasma reduced the glucose uptake to the level found in the absence of any added insulin.

Although it would be unlikely that, 3 months after her last known injection of insulin, exogenous insulin would still be present, this possibility was tested by giving her a small amount of insulin-I^{131}

[3] Dr. Gerold Grodsky, University of California Medical School, San Francisco, kindly performed these determinations.

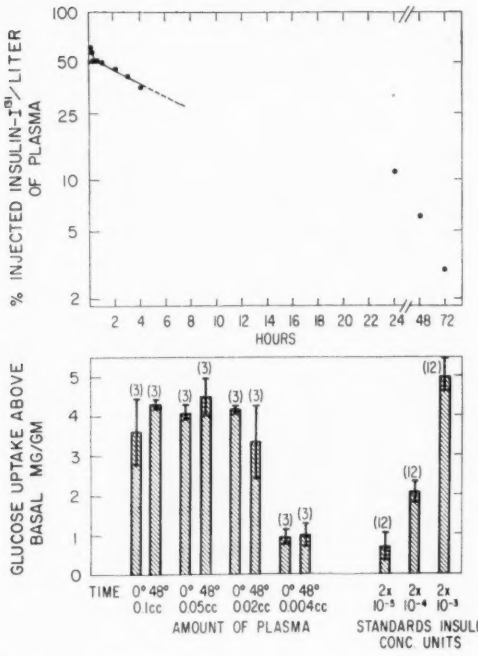

FIG. 4. COMPARISON OF PLASMA INSULIN ACTIVITY AND RATE OF DISAPPEARANCE OF INJECTED TRACER INSULIN-I^{131}.

and determining its rate of disappearance as well as the plasma insulin levels; 48 hours after injection, approximately 90 per cent of the insulin-I^{131} had left the plasma (Figure 4). Despite the marked decrease in insulin-I^{131} the insulin content of plasma obtained just before the insulin-I^{131} injection and 48 hours later was essentially unchanged. This was true with various amounts of plasma, since decreasing the amount of plasma to 0.02 ml did not appreciably diminish the stimulatory effect. It should be noted that the insulin standards employed in this study varied from 2×10^{-5} to 2×10^{-3} U as compared with 2×10^{-3} to 2×10^{-1} U in the two previous ones. From these studies it might appear that the insulin content of the plasma was greater than 20 mU per ml. This may not be the case, since it is known that dilution of plasma causes an apparent increase in its insulin activity (20).

In view of the large amounts of endogenous circulating insulin, it seemed feasible to attempt to fractionate the plasma to determine the location of insulin. Insulin-like activity has been reported in α-, β-, and γ-globulin fractions, by various fractionation procedures (21–23). When stimulation of glucose uptake by rat diaphragm was used as a measure of insulin action, most of the activity appeared in the zone between the γ- and β-globulins (Figure 5). In addition, there was a small peak of activity just behind albumin. When glycogen deposition was measured, insulin was found only in the region between the γ- and β-globulins and extending into the β-globulins (Figure 6). The fractions just behind albumin, which stimulated glucose uptake, caused no increased glycogen deposition. Since glycogen deposition was a less sensitive parameter of insulin action (Figures 1 and 2), the amount of insulin in the fractions just behind albumin might not be sufficient to be detected by this method. It was also possible that these fractions contained other non-insulin substances which stimulated glucose uptake. It is known that several substances increase glucose uptake by rat diaphrgam, but insulin appears to be unique in its ability to augment glycogen content (24, 25). However, with insulin antagonist plasma, it was possible to abolish the glucose uptake stimulatory effect by the fractions that migrated just behind albumin, which suggests their identity with insulin.

Since this patient had received exogenous insulin injections for over 1 year, her plasma should

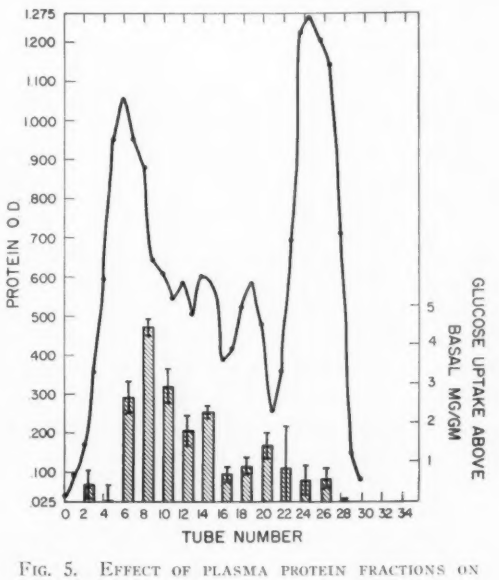

FIG. 5. EFFECT OF PLASMA PROTEIN FRACTIONS ON GLUCOSE UPTAKE BY ISOLATED RAT DIAPHRAGM.

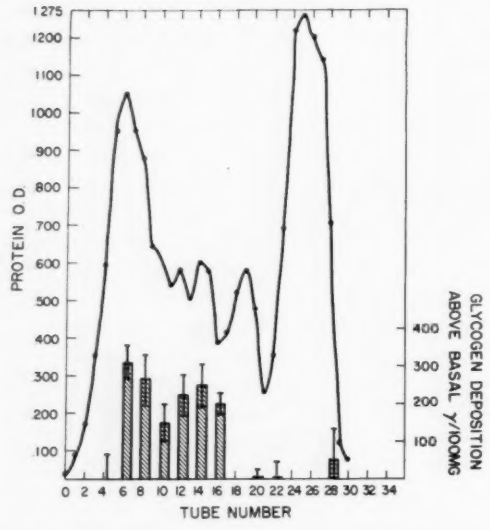

FIG. 6. EFFECT OF PLASMA PROTEIN FRACTIONS ON GLYCOGEN DEPOSITION BY ISOLATED RAT DIAPHRAGM.

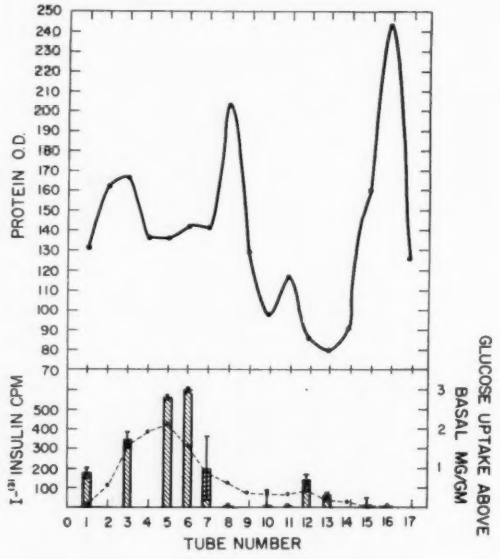

FIG. 7. CORRELATION BETWEEN ELECTROPHORETIC MIGRATION OF ADDED INSULIN-I^{131} AND INSULIN ACTIVITY OF POU PLASMA.

contain insulin-binding antibody, as demonstrated by Berson and co-workers (14). This insulin-binding protein is located in the region between the γ- and β-globulins and thus it is difficult to know whether the insulin activity in her plasma in this region represents insulin bound to the antibody or whether this is where endogenous insulin normally resides. When normal plasma with added insulin was fractionated in this manner, the insulin was recovered just behind albumin (14). It might be that the small amount of insulin in the patient's plasma migrating behind albumin was free insulin, while the bulk of the activty in the inter-β-γ region was bound. A small amount (20 μU per

TABLE II

Effect of insulin on glucose uptake by human adipose tissue

Patient	Insulin concentration/ml			
	0	0.1 U	0.5 U	1 U
Normal	2.16* ± 0.13 [4]†	3.16 ± 0.15 [2] p < 0.01‡		5.08 ± 0.13 [2] p < 0.01
POU	0.85 ± 0.37 [4]	1.21 ± 0.28 [4]	1.73 ± 0.13 [4]	3.34 ± 0.15 [4] p < 0.01
Juvenile diabetic	2.16 ± 0.52 [4]	2.11 ± 0.14 [4]	4.16 ± 0.37 [4] p < 0.02	4.18 ± 0.21 [4] p < 0.02
Obese diabetic	1.69 ± 0.17 [4]	1.57 ± 0.20 [4]	3.08 ± 0.41 [4] p < 0.05	3.52 ± 0.20 [4] p < 0.01
Chronic insulin-resistant diabetic	0.91 ± 0.19 [4]	1.46 ± 0.38 [4]	2.79 ± 0.22 [4] p < 0.01	3.45 ± 0.10 [4] p < 0.01

* Expressed as mg/g ± SE of the mean.

† [ ], Number of determinations.

‡ p Value calculated on the comparison of the glucose uptake in absence and presence of insulin. Effect of insulin was not significant when no p value is given.

ml) of insulin-I^{131} was added to her plasma before fractionation to determine whether the bound insulin-I^{131} would correspond to the area of endogenous insulin activity. Figure 7 demonstrates that most of the insulin-I^{131} was found between the β- and γ-globulins in the same region with the bulk of the insulin activity. The amount of stimulation of glucose uptake was greatly in excess of the small amount of insulin-I^{131} added to the plasma prior to electrophoresis. In addition, there was a very small peak of insulin-I^{131} just behind albumin, coinciding with the fractions that also appeared to contain some insulin.

The finding of large amounts of endogenous insulin in the patient's plasma in the presence of demonstrated unresponsiveness to its hypoglycemic effect certainly suggests absent or decreased tissue response to the hormone. The effect of insulin on her adipose tissue was tested in an attempt to obtain direct proof of this (Table II). When insulin was added to adipose tissue slices from a nondiabetic patient, there was a significant increase in the glucose uptake when 0.1 U per ml was used and an even greater effect with 1 U per ml. However, in contrast, the adipose tissue from this patient showed no significant response to the addition of 0.1 or 0.5 U per ml, but did demonstrate an increased glucose uptake when 1 U per ml was added. Adipose tissue from other representative diabetics, including one patient with chronic insulin resistance with circulating antagonist, also failed to respond to 0.1 U insulin per ml, but all gave significant increases when 0.5 U was present. The insulin effect on leukocytes was also measured in an attempt to assess peripheral tissue responsiveness. The data in Table III indicate that the patient's leukocytes responded normally to the large amounts of insulin used. Even during an episode of diabetic acidosis, when her leukocytes removed considerably less glucose from the medium than did normal cells, the response to added insulin was unimpaired.

TABLE III

Effect of insulin on leukocyte glucose uptake *

Source of leukocytes	Control	Insulin
	mg glucose/10^{10} WBC	
Patient pre-R_x diabetic acidosis	44	380
Patient postrecovery acidosis	154	431
Normal	169	323
Normal	142	360

* 0.6 U insulin/ml incubation medium; incubations for 4 hours.

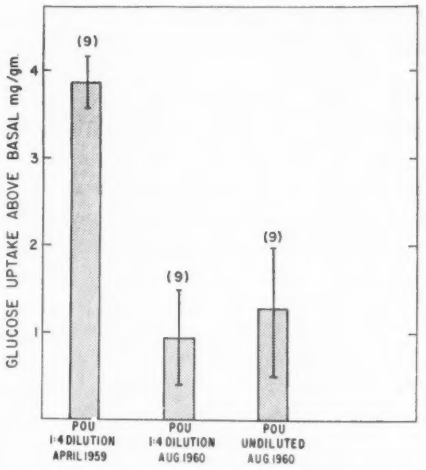

FIG. 8. COMPARISON OF INSULIN ACTIVITY IN PLASMA DURING PERIOD OF INSULIN UNRESPONSIVENESS AND LATER DURING REMISSION.

Whereas her plasma insulin levels were elevated during this period of unresponsiveness to insulin, at the time of her last admission, when the diabetes had remitted, her plasma insulin activity was normal (Figure 8).

During the initial Clinical Center admission, when she exhibited both diabetes and insulin unresponsiveness, her ability to oxidize glucose-1-C^{14} was assessed. Over a 5-hour period, 14.3 per cent of the tracer dose was recovered in $C^{14}O_2$. In similar studies, nondiabetics were found to oxidize an average of 37 per cent of the injected dose.

DISCUSSION

Clinically there can be little question that initially this patient was unresponsive to the hypoglycemic effects of insulin. Intravenous injection of as much as 200 U of glucagon-free insulin failed to depress the blood sugar to normal levels and caused only a slightly greater fall than did 10 U intravenously (Table I). Although she was maintained without insulin therapy, she had no diabetic symptoms despite glycosuria of 300 g per day on a 3,000 caloric diet. A diagnosis of diabetes certainly was justifiably based on her hyperglycemia and glycosuria. During her first admission to

the Clinical Center she also had constant acetonuria and three episodes of mild diabetic acidosis. During the first two of these she was treated with massive doses of insulin while she recovered from the third episode with fluid therapy alone. The role of such fluids in her recovery is difficult to assess, since there appeared to be no dramatic effect on the levels of the free fatty acids. Such spontaneous recovery from diabetic acidosis does not coincide with our usual concept of this condition.

It is even more surprising that she was not always in diabetic acidosis in view of her extreme insulin unresponsiveness and massive glycosuria. Her constant ketonuria suggested that oxidation of fat was providing most of her energy requirements and that her carbohydrate utilization was severely impaired. She was given a tracer dose of glucose-1-C^{14} and its oxidation to $C^{14}O_2$ was measured to assess this. Over a 5-hour period 14.3 per cent of the injected dose appeared as $C^{14}O_2$, compared with 34 to 46 per cent when normal subjects were studied (17). However, when one corrects for the patient's enlarged glucose pool and urinary loss of glucose-C^{14} during the 5-hour test period, it is apparent that she was able to oxidize almost as much glucose as the normal, although at a much higher blood glucose concentration. This ability of the diabetic to utilize as much glucose as the nondiabetic, but at a substantially raised blood glucose concentration, has been previously demonstrated (26). At the present time it is difficult to reconcile the marked ketonuria with the concept that she was oxidizing almost as much glucose as the nondiabetic. Even more difficult to explain than the absence of constant diabetic acidosis is the absence of any of the usual symptoms of diabetes. It is conceivable that, although she appeared quite insulin-resistant, she was obtaining a small effect as a result of her high plasma insulin levels, and this was enough to prevent acidosis. During her second hospital admission she did not demonstrate acetonuria, and she had actually gained weight despite her impressive glycosuria. In retrospect, she may have been starting the remission which was complete several months after her discharge. This would be substantiated by the observation that her glycosuria had decreased from approximately 300 g during her first Clinical Center admission to an average of 100 g during her second admission, with no change in diet. Her spontaneous remission was quite unexpected and its cause is completely unknown. At present her glucose tolerance is normal, with a tendency toward hypoglycemia several hours after glucose ingestion. It is impossible to determine whether this hypoglycemia represents a manifestataion of "pre-diabetes" (27) or reflects the residual hyperfunction of her islets which was thought to be present during the period of insulin-unresponsive diabetes. Although temporary remissions have been frequently observed soon after the diagnosis and energetic treatment of diabetes mellitus in some juvenile patients, remission of such severe diabetes as the present patient had is most unusual (9). Since the genesis of her diabetes and insulin resistance is obscure, it is impossible to elaborate on its exodus.

In addition to undergoing remission, this patient differs from other cases of insulin resistance presumed to be due to tissue unresponsiveness (5–8) in that she had greatly increased amounts of endogenous insulin in her plasma. In the previous cases, plasma insulin was demonstrable only at a time when the patient was receiving injections of large amounts of insulin. This patient still had increased plasma insulin 3 months after her last known insulin injection. The fact that her plasma caused an *in vitro* increase in glycogen deposition of the isolated rat diaphragm as well as augmented glucose uptake strongly suggests that the activity was due to insulin. Although increased glucose uptake is probably not specific for insulin, this hormone is unique in its *in vitro* ability to stimulate glycogen deposition (24, 25). Salicylates and phenethylbiguanide also stimulate glucose uptake by diaphragm, but they both lead to a decrease in muscle glycogen. Further evidence for the identification of the activity as insulin is derived from the observation that its effect was abolished by the addition of plasma known to contain an insulin antagonist, presumably an antibody. This plasma had no effect on the basal uptake of glucose by the rat diaphragm but was capable of abolishing the increased glucose uptake produced by either insulin or the patient's plasma. In addition, assay of the patient's plasma by a specific immunological assay for insulin gave values in the same range as those obtained by bioassay. At a time when she was in remission her plasma insu-

lin activity was normal. This is consistent with her demonstrated responsiveness to both insulin and tolbutamide (Orinase).

Although the patient's last known insulin injection was 3 months prior to these studies, it was still possible that the insulin detected in her plasma was a residue from the large amount of exogenous insulin she was given. The results of the study in which the rate of disappearance of a tracer dose of insulin-I^{131} was followed make this unlikely. Whereas 90 per cent of the injected tracer had disappeared from the plasma at the end of 48 hours, the levels of plasma insulin were essentially the same as before the insulin-I^{131} was given. If the plasma insulin were exogenous in origin, one might expect the insulin concentration to decrease at the same rate as the insulin-I^{131} disappeared from the plasma. Since it did not, it indicates that pancreatic production of insulin must be continuing at a rate necessary to maintain the plasma level.

Another but less likely possibility is that the insulin-I^{131} did not label the entire insulin pool but was being metabolized at a rate different from that of a larger unlabeled insulin pool. Since this patient had demonstrable insulin antibody, one might consider the larger, unlabeled insulin pool as complexed with antibody, and the smaller, labeled pool as "free" insulin. It might thus be possible to maintain a constant plasma insulin level in association with a decreasing specific activity without implicating increased pancreatic insulin secretion if the rate of dissociation of insulin from antibody were considerably slower than the rate of disappearance of "free" insulin from the plasma. Against this possibility is the observation that the half-time disappearance of insulin-I^{131} was greatly prolonged, while in the nondiabetic, in the absence of insulin-binding antibody, it is about 30 minutes (14). This suggests that in this patient there was some, if not complete, mixing of insulin-I^{131} with the insulin-antibody pool. If the insulin-I^{131} were labeling only a smaller "free" insulin pool, one would expect its half-time of disappearance to approach that of the normal. The prolonged insulin-I^{131} disappearance in the insulin-treated subject has been presented as evidence for the existence of insulin-antibody complex and a reaction between injected insulin-I^{131} and insulin antibody (14). It also seems unlikely that the insulin-antibody dissociation would be so slow that it would be a factor 3 months after the patient's last insulin injection.

Hyperglycemia is a known stimulus for pancreatic insulin production (28), and since this patient had continuous hyperglycemia her pancreas was being continually stimulated despite increased plasma insulin levels. This finding again points to an underlying failure of tissue response to insulin. In contrast to the slow rate of insulin-I^{131} disappearance when she was resistant, the hormone disappeared normally when she was again responsive to insulin. During this latter period she had not received insulin for well over a year, so that the shorter half-life might be due to the loss of insulin antibody which would retain injected insulin in her plasma.

In this case and several others (5–8), the cause of the insulin resistance was presumed to reside at the tissue level. It was not possible to detect any excess of insulin antagonists in this patient's plasma. The insulin that was present in her plasma appeared to have biologic activity in that it increased glucose uptake and glycogen deposition in the isolated rat diaphragm and produced hypoglycemia in fasted mice.[4] When the response of her adipose tissue to insulin was studied, it was found less sensitive than adipose tissue from a normal person or other diabetics. This unresponsiveness was not complete, since in the presence of 1 U per ml of incubation medium, her response was undiminished. While 0.5 U per ml produced a significant increase in glucose uptake in all other patients studied, it failed to do so in the present patient. It is realized that these amounts of insulin are quite large and not in the physiologic range, making interpretation of the results more difficult. Nonetheless, there was a distinction between this patient's adipose tissue and that obtained from other diabetics, including one with chronic insulin resistance. However, the patient's leukocytes appeared to be as insulin-responsive as normal leukocytes when glucose uptake was measured. Since the amounts of insulin used in these experiments were also large, interpretation of the results in terms of *in vivo* responsiveness to insulin is difficult. It is of interest that except when the patient was in diabetic

[4] Dr. Frederick Goetz, University of Minnesota, kindly performed these studies.

acidosis, her leukocytes removed as much glucose from the incubation medium as do normal white blood cells. In the presence of a decreased tissue responsiveness to insulin, the greatly elevated plasma levels may have been sufficient to prevent severe diabetic acidosis. Although conclusive demonstration is lacking, the cause of her insulin resistance seems to be due to a decreased tissue response to this hormone.

By rat hemidiaphragm assay, her plasma contained between 2 and 20 mU of insulin per ml, while the normal level by a more specific immunoassay was approximately 0.02 mU per ml (29). Her plasma contained 10 mU per ml assayed by its hypoglycemic action in the fasted mouse.[4] Fractionation of the plasma by electrophoresis indicated that most of the insulin was present in the area between the β- and γ-globulins, while a small part migrated just behind albumin. Insulin was found in the β-globulins in another patient with insulin resistance in whose plasma exogenous insulin was detectable (6). In normal plasma, insulin has been reported to be located in the γ-, β- and α-globulins (21–23). Since insulin-binding protein also migrates in the area between γ- and β-globulin, and since this patient had received prior exogenous insulin, it is impossible to decide whether the insulin in the inter-γ-β zone was complexed to the insulin-binding protein or not. The former possibility might seem more reasonable, since there was also a small amount of insulin just behind albumin where "free" insulin is known to migrate (14).

SUMMARY

The case of an 18 year old Negro female diabetic with severe insulin unresponsiveness, presumably as a result of decreased tissue responsiveness, is presented. Over a period of 18 months there was a complete remission of both her insulin resistance and her diabetes. Unique features of the case include the presence of large amounts of circulating endogenous insulin which appeared to have full physiologic activity in the isolated rat hemidiaphragm and the fasted mouse at a time when she was unresponsive to the hormone. Its identification as insulin was based on its ability to increase *in vitro* muscle glycogen deposition as well as glucose uptake by rat diaphragm. In addition, its effect could be abolished by the addition of insulin antagonist plasma. While 90 per cent of an injected tracer dose of insulin-I^{131} disappeared from the plasma in 48 hours, there was no change in the plasma insulin level during this time. Fractionation of the plasma indicated that most of the insulin activity resided in the inter-γ-β-globulin zone, although there was also a small amount migrating just behind albumin. Adipose tissue obtained from the patient demonstrated no increase in glucose uptake in the presence of 0.1 and 0.5 U insulin per ml, while normal adipose tissue responded to the former and adipose tissue from other diabetics responded to the latter concentration. In the presence of 1 U per ml insulin, her adipose tissue demonstrated a normal response.

Although the cause of her insulin resistance was thought to be due to decreased tissue responsiveness, the mechanism of this as well as her subsequent complete remission, is unknown.

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THE METABOLISM OF TRITIATED FOLIC ACID IN MAN*

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Several microbiological methods are available for the assay of folic acid and related coenzymes in tissues and body fluids (1, 2). Many conjugated forms of folic acid are not detected by such assays unless first converted enzymatically to active forms (3). The presence of folic acid antagonists or of antibiotics can also render difficult the quantitative determination of folic acid and its conjugates by present microbiological techniques. In order, therefore, to facilitate the study of the metabolism of folic acid in normal subjects and in patients with hematological and other disorders, tritiated folic acid has been prepared and some aspects of its metabolism in man studied by non-microbiological assay methods. The present paper presents the results of these studies, preliminary accounts of which have appeared (4, 5).

METHODS AND MATERIALS

Preparation of tritiated folic acid

Finely powdered USP grade folic acid, 500 mg, was exposed to 15 c tritium at 27°C and 0.4 atm pressure for 10 days. The crude tritiated material had an activity of 220 μc per mg; 40-mg batches of this material were dissolved in 0.2 per cent $NaHCO_3$ and chromatographed in the dark at room temperature on a 2×20 cm column packed with DEAE-cellulose (Eastman Kodak 7392) equilibrated by the method of Sober, Gutter, Wyckoff and Peterson (6). Elution was started with 0.01 M sodium phosphate buffer at pH 6.9. At 280 ml, buffer strength was raised to 0.2 M. A flow rate of 2 ml per minute was used. The effluent was passed through an ultraviolet absorptiometer (Uvicord, LKB, Stockholm) and collected in 10-ml portions in an automatic fraction collector (RadiRac, LKB, Stockholm). The absorptiometer lamp was switched off during the passage of the folic acid peak to minimize photochemical decomposition. Major impurities were found by ultraviolet analysis of the fractions to be 2-amino-4-hydroxy-6-methylpteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid, *p*-aminobenzoylglutamic acid and pteroic acid. The folic acid fractions were pooled and rechromatographed. The specific activity of the folic acid was found to be 12.2 ± 0.2 μc per mg. Additional chromatographic purification did not further lower this value. In order to minimize radiation decomposition, a weight of glucose 20 times that of the weight of the pooled folic acid was added and the resulting folic acid-sodium bicarbonate-glucose solution lyophilized and stored in the dark at -5°C. The microbiological activity of this preparation of tritiated folic acid was determined by *Lactobacillus casei* assay by the method of Baker and co-workers (2) and was equal to that of an equivalent weight of USP folic acid.

Distribution of radioactivity within the folic acid molecule. Two mg of tritiated folic acid of specific activity 1.40×10^{10} dpm per mmole was dissolved in 2 ml of 0.2 per cent $NaHCO_3$; 3 ml of 2 N H_3PO_4 and 200 mg of zinc were added and reduction allowed to proceed for 30 minutes; 600 mg of $CaCO_3$ was then added and the mixture left to stand overnight. The clear supernatant was decanted and applied to a column of DEAE-cellulose, 1×7.5 cm. Elution was started with 0.01 M sodium phosphate buffer, pH 6.9, the effluent being passed through an ultraviolet absorptiometer. After the passage of the 2-amino-4-hydroxy-6-methylpteridine peak, buffer strength was increased to 0.2 M. The pteridine and *p*-aminobenzoylglutamic acid fractions were collected. No unsplit folic acid was detectable in the effluent. The concentration of *p*-aminobenzoylglutamic acid was determined by the Bratton-Marshall method (7), and the concentration of 2-amino-4-hydroxy-6-methylpteridine by the measurement of ultraviolet absorption at 252 mμ and 363 mμ, at pH 13. The specific activity of the *p*-aminobenzoylglutamic acid was 1.06×10^{10} dpm per mmole or 75.7 per cent that of the starting material, and the specific activity of the pteridine was 0.31×10^{10} dpm per mmole or 22.1 per cent that of the starting material; 97.8 per cent of the radioactivity present in the folic acid was therefore accounted for after reductive cleavage.

To 0.5 mg of tritiated *p*-aminobenzoylglutamic acid in 9.5 ml of 0.2 M sodium phosphate buffer collected by the foregoing procedure was added about 9.5 mg of nontritiated *p*-aminobenzoylglutamic acid. An aliquot was taken for the determination of specific acivity, which amounted to 5.07×10^8 dpm per mmole. Sufficient concentrated H_2SO_4 was added to bring the solution to 2 N. Hydrolysis was allowed to proceed for 10 hours at 100°C. The reaction mixture was then brought to 50 ml with distilled water and allowed to stand overnight with a large excess of $BaCO_3$.

i) Ten ml of the reaction mixture was brought to pH 3 with 5 N HCl and extracted with 10 ml of ethyl ace-

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† Research Fellow of the National Cancer Institute of Canada.

tate. The extract was then taken to dryness and the residue redissolved in 3 ml distilled water. The solution was chromatographed on DEAE-cellulose and the *p*-aminobenzoic acid fraction collected. No α-amino nitrogen was detectable by the ninhydrin reaction. The concentration was determined by the Bratton-Marshall method. The specific activity was 1.43×10^8 dpm per mmole or 28.2 per cent that of the starting material.

ii) Ten ml of the reaction mixture was chromatographed on DEAE-cellulose and the *p*-aminobenzoic acid (PABA) and *p*-aminobenzoylglutamic acid fractions collected. The PABA fraction was brought to pH 3 with 5 N HCl and extracted with 10 ml of ethyl acetate. The extract was washed with 5 ml distilled water and the washings discarded. The ethyl acetate extract was then taken to dryness and the residue redissolved in 5 ml distilled water. No α-amino nitrogen was detectable by the ninhydrin reaction. The concentration of *p*-aminobenzoic acid was determined by the Bratton-Marshall method. The specific activity was 1.45×10^8 dpm per mmole or 28.6 per cent that of the starting material.

The distribution of radioactivity in this preparation of tritiated folic acid is therefore 22.1 per cent in the pteridine fraction, probably on carbon 9, 28.4 per cent in the *p*-aminobenzoic acid, and 47.3 per cent in the glutamic acid. These together account for 97.8 per cent of the total radioactivity.

Twenty-four hours prior to use, the lyophilized folic acid was redissolved in distilled water and rechromatographed. The effluent was precipitated at pH 3 with 5 N HCl, separated by centrifugation, and redissolved in sufficient 0.9 per cent NaCl containing 0.2 per cent $NaHCO_3$ to give a concentration of 200 μg per ml. One per cent benzyl alcohol was added and the solution passed by means of a Swinny syringe adapter (Millipore Filter Corp., Bedford, Mass.) through a Millipore HA filter into a sterile vial. An aliquot was taken for determination of the specific activity and the solution kept in the dark at 4°C until immediately before use. Solutions for oral administration were prepared in the same way except that sterilization was omitted.

Tritiated folic acid prepared by exchange with tritiated water. Seven-tenths mg of tritiated folic acid prepared by an exchange reaction with tritiated water (TRA. 34, batch 5, Radiochemical Centre, Amersham, England) was purified by chromatography on DEAE-cellulose. The major impurities were *p*-aminobenzoylglutamate and two unidentified pteridines. The specific activity after purification was 174 μc per mg. Solutions were prepared for administration as described above.

A similar preparation was used by Anderson, Belcher, Chanarin and Mollin (8) in studies of the handling of orally administered tritium-labeled folic acid in patients with megaloblastic anemias; these workers found that radioactivity was distributed almost equally between the *p*-aminobenzoylglutamate and the pteridine portions of the molecule.

Tritiated folic acid prepared by exchange with tritiated acetic acid. Twenty mg of folic acid USP was shaken with 15 mg of pre-reduced platinum catalyst and acetic acid containing 25 c tritium at 100°C for 12 hours. After removing catalyst and acid, the labile tritium was removed by dissolving the residue in 0.1 per cent $NaHCO_3$ and precipitating the folic acid at pH 3 with dilute HCl. The activity of the crude tritiated material was 62 μc per mg. Purification was carried out by chromatography on DEAE-cellulose, the major impurities being *p*-aminobenzoylglutamate and an unidentified pteridine. The specific activity was 52 μc per mg.

In the experiments described below, the preparations of tritiated folic acid used were those labeled by exchange with tritium gas and by exchange with tritiated water. No differences in *in vivo* behavior between the batches of labeled folic acid prepared by the two different methods were noted. Folic acid labeled by the third method, exchange with tritiated acetic acid, was not used in this study, but preliminary experiments suggest that it, too, is handled by the body in the same way.

Procedures

1. Assay of plasma tritiated folic acid. i) Isolation method. To 5 ml of heparinized plasma containing an unknown amount of tritiated folic acid was added 0.5 ml of a 0.2 per cent solution of nontritiated folic acid in 0.2 per cent $NaHCO_3$; 5.3 ml of acetone (reagent grade, Fisher) was added, the solution mixed and centrifuged at 3°C and 2,500 rpm for 10 minutes, and the supernatant decanted into a 50 ml polyethylene test tube; 25 ml of acetone was added, the solution mixed and centrifuged in the cold and the supernatant discarded. The precipitate was redisolved in 3 ml distilled water and poured into a 15 ml centrifuge tube. The polyethylene tube was washed once with 3 ml of 0.2 M phosphate buffer at pH 6.9 and the washings added to the centrifuge tube. The folic acid was precipitated at pH 3 by the addition of 5 N HCl and separated by centrifuging at 3°C. The supernatant was discarded. The precipitate was redissolved in 1 ml of 0.2 per cent $NaHCO_3$; 150 mg of zinc and 1 ml of 2 N H_3PO_4 were added and reductive cleavage allowed to proceed for 2 hours; 200 mg $CaCO_3$ was added and the solution allowed to stand overnight. The solution was then centrifuged and the supernatant decanted into a 15 ml centrifuge tube. The precipitate was washed once with 2 ml of water and the washings added to the supernatant. The supernatant was kept at 80°C for 20 minutes in the oven, recentrifuged and decanted. One-tenth ml of this solution was taken for measurement of diazotizable amine by the Bratton-Marshall method (7). The total folic acid could be calculated from the value obtained. Three ml was added to counting vials and taken almost to dryness in the oven at 80°C and then to complete dryness in a vacuum desiccator; 1 ml of a 10 per cent solution of Hyamine in toluene was added and the vials allowed to stand for several hours until solution was complete; 5 ml of scintillator was then added and the tritium concentration determined in the liquid scintillation counter. Efficiencies averaged 14.2 per cent.

In some cases, all nonfolic acid radioactivity was removed by redissolving the folic acid precipitated at pH 3 during the isolation procedure in 0.2 per cent $NaHCO_3$

TABLE 1

Recovery of tritium-labeled folic acid added to plasma and urine

	H^3-folic acid added	Carrier folic acid added	Carrier folic acid after isolation	H^3-folic acid after isolation	Original H^3-folic acid	Recovery
	dpm	*μg*		*dpm*		%
Isolation method, plasma	6,000	1,000	438	2,695	6,145	102.4
	6,000	1,000	414	2,536	6,137	102.3
	6,000	1,000	438	2,640	6,019	100.3
	1,900	1,000	453	842	1,860	97.9
	1,900	1,000	426	784	1,842	96.9
	1,900	1,000	399	764	1,918	100.9
Direct chromatography, urine	5,000	364	120	1,606	4,870	97.3
	5,000	364	100	1,373	4,998	100.0
	5,000	364	107	1,563	5,320	106.4
	22,000	364	96	6,012	22,785	103.6
	22,000	364	84	5,093	22,053	100.2
	22,000	364	92	5,711	22,616	102.8
Isolation method, urine	33,000	1,143	360	10,317	32,808	99.4
	33,000	1,143	354	10,152	32,791	99.4
	33,000	1,143	402	11,260	32,050	97.2
	8,400	1,143	102	738	8,273	98.5
	8,400	1,143	132	994	8,608	102.5
	8,400	1,143	393	2,868	8,346	99.6

and purifying it by column chromatography on DEAE-cellulose. The collected folic acid fraction was then reprecipitated at pH 3 and the usual procedure followed. Nonfolic acid radioactivity ranged from 11 to 23 per cent of the total radioactivity measured by this method.

ii) Assay of total plasma radioactivity. Eight-tenths ml of heparinized plasma containing tritated folic acid was added to 15 ml of Thixcin-Hyamine emulsion and counted at $-5°C$ by the method of Shapira and Perkins (9). Standards of known amounts of tritiated folic acid in blank plasma were run; efficiencies averaged 5.4 per cent.

2. Assay of tritiated folic acid in the urine. i) Direct chromatography. To 5 ml of urine containing an unknown amount of tritiated folic acid was added 1 ml of a 0.2 per cent solution of nontritiated folic acid in 0.2 per cent $NaHCO_3$; 2 ml of this solution was then applied to a 0.5×5 cm column of DEAE-cellulose, followed by 20 ml of 0.01 M sodium phosphate buffer at pH 6.9. The folic acid, visible as a yellow band, was eluted with 0.2 M sodium phosphate buffer, pH 6.9. The folic acid was collected and precipitated at pH 3 with 5 N HCl. The precipitate was centrifuged, redissolved, reduced, chemically assayed and counted by the same method used for the plasma folic acid.

ii) Isolation method. For highly pigmented urines, direct chromatography as described above was not suitable. To these urines, carrier folic acid was added in the same way, and the total folic acid was precipitated at pH 3 with 5 N HCl and separated by centrifugation at 3°C. The precipitate was redissolved in 6 ml of 0.2 per cent $NaHCO_3$; 2 ml of this solution was then chromatographed as described above.

iii) Assay of total urine radioactivity. Five-tenths ml urine was added to a counting vial and taken to a volume of about 0.1 ml in the oven at 80°C; 0.4 ml Hyamine was then added, followed by 1 ml ethanol and 5 ml of scintillator. All samples were run in quadruplicate. As an alternative method, 1 ml of urine was added to a counting vial containing 3 g naphthalene (reagent grade, Matheson, Coleman, and Bell); 15 ml of scintillator was then added, made up as follows: 77 ml dioxane, 23 ml ethanol, 25 mg 1, 4-*bis*-2-(5-phenyloxazolyl)-benzene (POPOP) and 1 g 2,5-diphenyloxazole (DPO). The samples were counted at $-5°C$. Efficiencies averaged 7.2 per cent. If the deepfreeze temperature were raised, the addition of ethanol to the scintillator solution could be omitted with a consequent improvement in efficiency. This method was not suitable for concentrated urines containing a heavy sediment because of the adsorption of folic acid on the insoluble residue at the bottom of the counting vial and consequent low counts.

3. Assay of tritiated water in the urine. In a number of experiments water was distilled from the urine samples and assayed for tritium by the method of Werbin, Chaikoff and Imada (10).

4. Counting methods. A Packard TriCarb liquid scintillation counter was used for all tritium assays. Unless otherwise stated, samples were taken to dryness in 20-ml glass counting vials (Wheaton Glass Co., Millville, N. J.) and redissolved in 10 or 20 per cent Hyamine in toluene, prepared by the method of Eisenberg (11); 5 ml of

toluene containing 0.6 per cent DPO and 0.02 per cent POPOP (Pilot Chemicals Inc., Watertown, Mass.) was added and the samples counted with a discriminator window setting of 10 to 100, voltage at tap 6, and a deep-freeze setting of −5°C. Because of variations in the degree of quenching encountered, all samples were recounted after the addition of an internal standard (tritiated toluene, New England Nuclear Corp., Boston, Mass.). Efficiencies ranged from 5 to 14 per cent. Solutions containing nontritiated carrier folic acid were assayed chemically by the method of Bratton and Marshall (7).

5. Recoveries and reproducibility of results. The amount of tritiated folic acid originally present in those samples to which carrier folic acid was added, followed by an isolation procedure, was calculated as follows. If the amount of carrier folic acid added was x mg, the amount recovered (as measured by the Bratton-Marshall method) was y mg, and the tritium activity detected was z dpm, then the tritium activity present in the original samples was xz/y dpm. Reproducibility by this method is shown in Table I.

In those procedures in which radioactivity in urine and plasma was counted directly without isolation, the calculated amount of radioactivity present for the total plasma by the Thixcin gel method was 98.1 ± 2.1 per cent (mean of 10 trial determinations ± SD); for total urine radioactivity by the ethanol method, 99.5 ± 3.3 per cent (mean of 8 trial determinations ± SD); and for total urine radioactivity by the dioxane method, 101.9 ± 3.0 per cent (mean of 8 trial det rminations ± SD).

6. The stability of tritiated folic acid in plasma and urine. *i*) To centrifuge tubes containing 5 ml of heparinized plasma, 1.12 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added. The tubes were incubated for varying periods at 37°C, carrier folic acid added and the total folic acid then isolated and counted as described above. In 4 tubes in which carrier folic acid was added before incubation, recoveries were 100.4 ± 6.8 per cent (mean ± SD) but in 2 tubes in which the addition of carrier was delayed for 1 hour, recovery averaged 87.5 per cent, and in 2 tubes in which carrier was added at 3 hours, recovery averaged 88.8 per cent. In the experiments to be described, carrier folic acid was therefore added to the plasma immediately after collection.

ii) To 2-ml aliquots of a blank urine which had been allowed to stand overnight with $CaCO_3$, 1.34 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added (see above). The urines were then incubated for periods of 1, 6, and 24 hours, carrier folic acid was added and the total folic acid isolated by chromatography on DEAE-cellulose as described above. Recoveries did not decrease significantly with time.

iii) To six 2-ml aliquots of blank urine which had been brought to neutral pH with $CaCO_3$, 1.12 μg of tritium-labeled folic acid of specific activity 35,700 dpm per μg was added. Two aliquots were brought to pH 5.25 with 0.1 N HCl, two were brought to pH 8.1 with 0.1 N NaOH and two allowed to remain at neutrality. One urine from each group was then incubated for 3 hours at 37°C and the H^3-folic acid assayed by addition of carrier, followed by isolation on DEAE-cellulose. Recoveries were as shown in Table II.

TABLE II

Stability of tritium-labeled folic acid in urine at alkaline and acid pH

pH	Time of incubation	Recovery	Recovery
	hrs	*dpm*	*%*
5.25	0	38,720	96.8
5.25	3	32,950	82.4
6.9	0	41,010	102.5
6.9	3	39,890	99.7
8.1	0	39,100	97.8
8.1	3	33,235	83.1

Because of this apparent instability of small amounts of folic acid in urine at acid or alkaline pH, urines in the experiments described below were brought to neutrality and carrier folic acid added immediately after collection.

7. Administration of tritiated folic acid. The folic acid solution was injected into an antecubital vein by means of a pre-set delivery syringe through a 3-way stopcock and followed with 10 ml of normal saline. In most experiments a dosage of 15 μg per kg was used. Blood samples were taken into heparinized syringes from an antecubital vein of the other arm by an indwelling siliconed Cournand needle.

Experimental subjects

The subjects for this series of experiments were 16 healthy adult males, members of the Resident Staff of the Montreal General Hospital, ranging in age from 22 to 29 years and in weight from 59 to 86 kg. All were in a good state of nutrition. No subject was used for more than four experiments nor did any subject receive more than a total of 25 μc of tritium. At least 4 weeks was allowed to elapse between successive experiments on the same subject. The plasma disappearance curve of tritium-labeled folic acid after a standard dose of 15 μg per kg was determined for 10 of these subjects. The limits of variation were small; for instance, 30 minutes after injection the mean plasma concentration was 2.35 ± 0.25 per cent of the dose per L (mean ± SD); i.e., the coefficient of variation was 10.6 per cent.

RESULTS

After the intravenous administration of 1 μg per kg of tritiated folic acid to healthy adults, the concentration in the plasma falls very rapidly so that in a typical experiment, after 3 minutes only 2 per cent of the dose per L remained, and by 30 minutes only 0.1 to 0.2 per cent per L (Figure 1). This amount is equivalent to only about 0.1 μg per L. Thereafter the fall in concentration is slow and

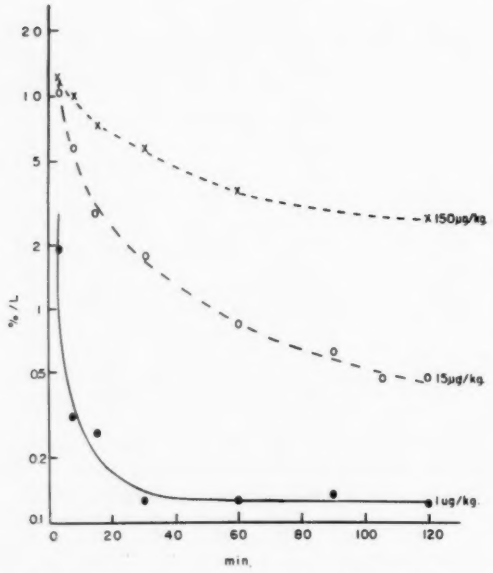

FIG. 1. PLASMA CONCENTRATION OF FOLIC ACID RADIOACTIVITY AFTER THE INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF 9 TO 14 μC TRITIATED FOLIC ACID DILUTED WITH CARRIER TO GIVE TOTAL DOSES OF 1, 15, AND 150 μG PER KG, RESPECTIVELY. Ordinate: % injected dose/L plasma.

difficult to follow with precision because of the extremely low levels of radioactivity involved (0.01 to 0.03 μc per L). This extremely rapid disappearance of tritiated folic acid from the plasma is not due to urinary excretion, since in the same experiment less than 2 per cent of the injected activity had been excreted in the urine by 2 hours (Figure 2) and the later excretion was even slower (less than 0.5 per cent per hour).

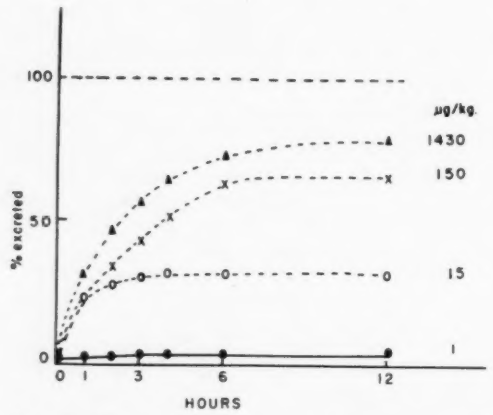

FIG. 2. URINARY EXCRETION OF RADIOACTIVITY AFTER THE INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF TRITIATED FOLIC ACID DILUTED WITH CARRIER TO GIVE TOTAL DOSES OF 1, 15, 150, AND 1,430 μG PER KG, RESPECTIVELY. Ordinate: cumulative excretion of radioactivity in the urine as a percentage of the injected dose.

When the dose of folic acid injected was increased to 15 μg per kg [1] the fall in plasma concentration was considerably slower, so that in a typical experiment 3.0 per cent of the dose was still present per L of plasma after 15 minutes and 0.5 per cent per L after 2 hours. In two subjects in whom satisfactory measurements were obtained 6 hours after injection, the residual fractions were 0.15 and 0.1 per cent per L, respectively. This level is therefore similar to that found after the lower dose, although it is attained about one-twentieth as fast and in absolute amount is equivalent to 1.3 to 1.6 μg per L of folic acid. With this higher dose of folic acid the urinary excretion of radioactivity is much greater (Figure 2) so that by the end of 2 hours, 20 to 30 per cent of the injected activity has been recovered in the urine; subsequently, the excretion rate falls steeply so that only 5 to 10 per cent more is excreted in the following 24 hours and it thereafter falls to less than 2 per cent per day.

Raising the dose to 150 μg per kg leads to a further slowing of the fall of plasma concentration and to a greater and more sustained urinary excretion. After 12 hours, 60 per cent of the radioactivity had been recovered in the urine (Figure 3). Increasing the dose of folic acid to 1,430 μg per kg had little additional effect on either the rate of disappearance of plasma activity or the urinary excretion.

In some experiments the total plasma radioactivity was also measured. When the plasma radioactive folic acid exceeded 100 μg per L, the radioactivity due to compounds other than folic acid was less than 5 per cent of the total. No determinations of nonfolic acid radioactivity were carried out at lower levels.

[1] Many of our studies have been carried out with a dose of 15 μg per kg in order to allow comparison with the extensive studies of Chanarin and co-workers (12–14) who used the same dose but a microbiological assay method. The agreement between the results obtained by the two methods is good. This dose is also convenient in that the plasma radioactivities are higher and thus easier to handle than the lower levels found with doses of about 1 μg per kg.

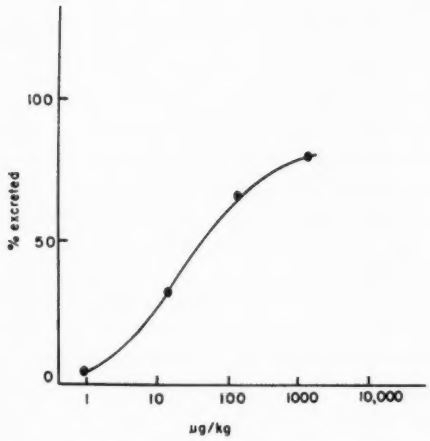

FIG. 3. THE RELATIONSHIP BETWEEN THE AMOUNT OF RADIOACTIVITY EXCRETED IN THE URINE UP TO 12 HOURS AFTER INTRAVENOUS INJECTION OF TRITIATED FOLIC ACID WHEN THE CARRIER MASS WAS VARIED FROM 1 TO 1,430 μG PER KG; 12 HOURS WAS CHOSEN AS THE ENDPOINT, SINCE AT THIS TIME THE RATE OF EXCRETION OF RADIOACTIVITY HAD FALLEN TO A LOW LEVEL.

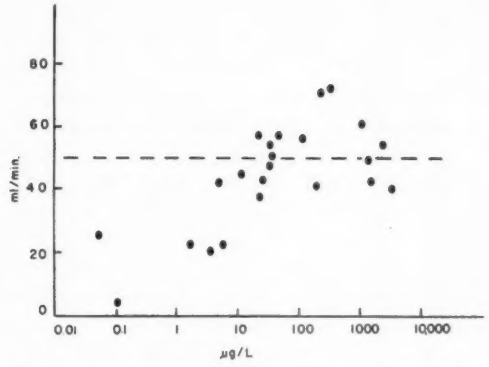

FIG. 4. URINARY CLEARANCE OF FOLIC ACID IN NORMAL SUBJECTS IN RELATION TO PLASMA CONCENTRATION. The dashed line is the clearance expected if filtration of the plasma folic acid that is not protein-bound occurred without tubular reabsorption or secretion. It agrees well with the mean clearance found when the plasma concentration was in excess of 10 μg per L. Ordinate: urinary clearance of radioactive folic acid. Abscissa: plasma radioactive folic acid.

A variable proportion of the radioactivity excreted in the urine was unchanged folic acid; this depended on the dose given. With 150 μg per kg, 75 per cent was folic acid; with 15 μg per kg, 40 to 50 per cent; and with 1.5 μg per kg, only 1 to 30 per cent. The identity of the other radioactive compounds present is dealt with below.

Renal handling of folic acid. During the course of several of the studies described in this report it was possible to collect urine during periods when the plasma radioactive folic acid concentration had been maintained for 1 hour or longer. The range of plasma concentrations studied was 0.05 to 3,000 μg per L.

At plasma concentrations above 10 μg per L the clearance is independent of the plasma level and averages 51 ± 3.1 ml per minute (Figure 4). At lower plasma concentrations the clearance falls. The finding of a fixed upper limit of clearance lower than the glomerular filtration rate (130 to 140 ml per min) made us suspect that a considerable fraction of the plasma folic acid was nonfiltrable because of binding to plasma proteins. This possibility was examined by ultrafiltration of plasma containing radioactive folic acid. The plasma was equilibrated at 37°C with CO_2 at a partial pressure of 40 mm Hg. The results illustrated in Figure 5 show that binding is practically uniform over the whole concentration range of 5 to 3,000 μg per L with an average of 64 per cent bound. Similar results were obtained by equilibrium dialysis.

Making allowance from these figures for the amount of folic acid that is bound and unavailable for filtration, we arrive at a clearance of 140 ml per minute for free folic acid. It appears, therefore, that the renal handling of folic acid for plasma levels above 10 μg per L can be accounted for simply by glomerular filtration of unbound folic

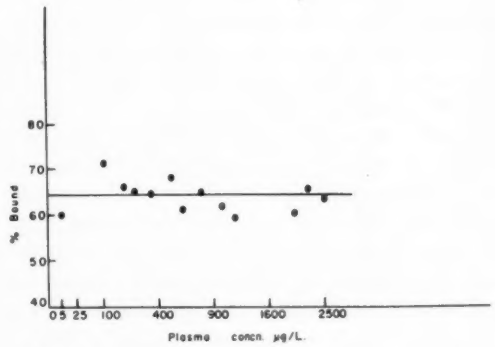

FIG. 5. PERCENTAGE OF RADIOACTIVE FOLIC ACID BOUND TO PLASMA PROTEIN AS DETERMINED BY ULTRAFILTRATION AT 37°C AND 40 MM HG pCO_2.

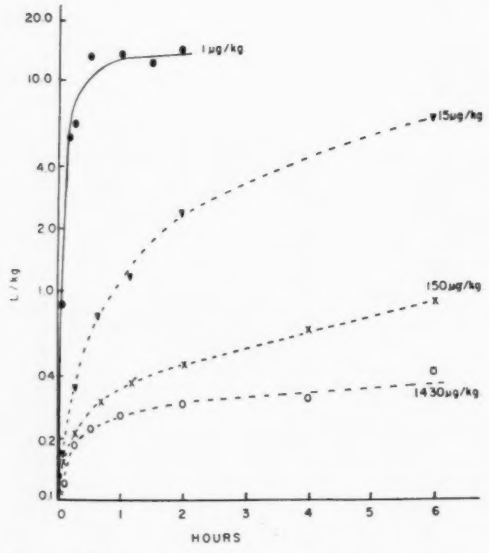

FIG. 6. VOLUME OF DISTRIBUTION OF RADIOACTIVE FOLIC ACID RETAINED IN THE BODY AFTER INTRAVENOUS INJECTION OF 9 TO 14 µC OF TRITIATED FOLIC ACID DILUTED WITH CARRIER TO 1, 15, 150, AND 1,430 µG PER KG, RESPECTIVELY. The extracellular water was taken to be 0.16 L per kg and the total body water 0.56 L per kg. When the carrier dose was 15 µg per kg or less, the radioactivity was rapidly distributed into a space larger than the total body water.

acid without appreciable modification by the tubules. At lower plasma concentrations, however, the clearance is lower than would be predicted from these considerations and probably involves a low rate of tubular reabsorption—not exceeding 0.03 µg per minute. The possibility must be considered that this represents metabolic usage of folic acid by the renal tubules or urinary excretory passages rather than a renal conservation process.

Disposition of retained activity. Calculation of the volume of distribution of the retained radioactivity shows that with doses of 1 to 15 µg per kg it rapidly exceeds the volume of the total body water (ca 0.56 L per kg; Figure 6). Indeed, if the amount of folic acid retained in the plasma and extracellular fluid is calculated (after due allowance for plasma protein binding) and compared with the total activity retained, with a dose of 1 µg per kg by 2 hours it is only 1/150 of the total; with 15 µg per kg at the same time it is 1/27; while for 150 µg per kg it is 2/9.

However, while the distribution of folic acid gives evidence of the rapidity with which radioactivity is taken up into the intracellular space, it does not necessarily show that folic acid itself is concentrated within the cells, since it is possible that it might be trapped within the cells by conversion to a nondiffusible metabolite. A further alternative might be rapid metabolism of folic acid to biologically inactive products. Any extensive metabolism might be expected to yield tritium to the body-water pool. This last possibility seemed improbable because the technique of whole-plasma counting by the emulsion method should be able to detect such products but did not show adequate activity to account for any such metabolic conversion. However, to settle the matter, urine samples were distilled and the radioactivity in the water measured by the method of Werbin and colleagues (10); 24 hours after the administration of 15 µg per kg (10 µc) of folic acid, no radioactivity was detectable in the urinary water (i.e., < 0.01 µc per L), whereas complete metabolism of the 6 to 7 µc of retained activity should have given about 0.15 µc per L of tritium in the body water. We were therefore led to the conclusion that the uptake of folic acid did not involve extensive degradation.

On the other hand, if folic acid itself were being concentrated within the cells, the lower intracellular : extracellular ratio found when large amounts of folic acid are administered suggests that it might be possible to displace activity from the cells by subsequent administration of a large carrier dose of nonradioactive folic acid. This proved to be the case. Figure 7 shows an experiment in which 1.55 µg per kg of tritiated folic acid was given; by 6 hours the plasma radioactive folic acid had fallen to 0.13 per cent of the dose per L and a total of 6.5 per cent of the injected activity had been excreted in the urine. At this time 450 µg per kg of nonradioactive folic acid was injected intravenously; this was followed by a rapid rise in plasma radioactivity to 3 per cent of the dose per L and a greatly increased excretion of radioactivity in the urine. In the next 12 hours a further 27 per cent of the injected radioactivity was excreted, of which 86 per cent was identified as folic acid. In comparable experiments with a dose of 15 µg per kg of radioactive folic acid, results were similar except that as much as 90 per cent of the radioactivity was recovered in 24 hours. In this case also most of the urinary radioactivity

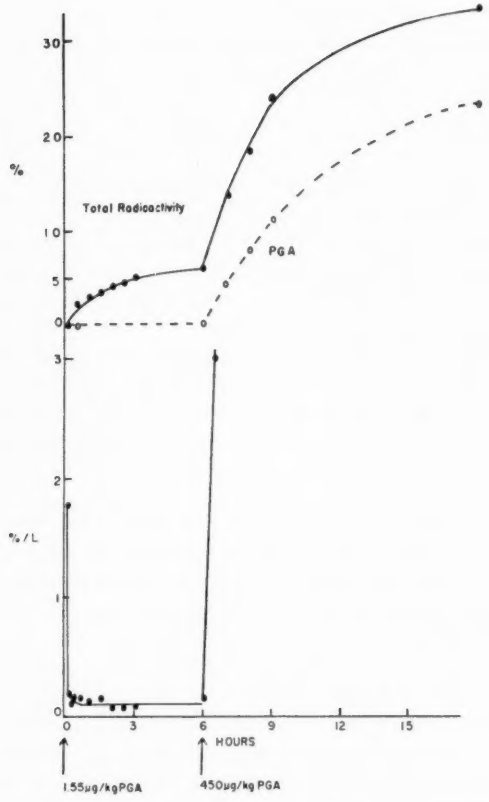

FIG. 7. NORMAL SUBJECT GIVEN 25 µC OF TRITIATED FOLIC ACID INTRAVENOUSLY (TOTAL FOLIC ACID 1.55 µG PER KG). Plasma radioactive folic acid, and urinary radioactive folic acid and total radioactivity were followed. Six hours after the first injection 450 µg per kg of nonradioactive folic acid was injected i.v. This was followed by a prompt rise in plasma radioactive folic acid and in the rate of urinary excretion. Lower curve: plasma folic acid radioactivity as percentage of dose injected. Upper curves: solid line, total radioactivity excreted; dashed line, radioactive folic acid excreted.

was identified as folic acid. At this dose-level, experiments were done in which intervals of up to 3 days elapsed between administration of the tritiated folic acid and the "flushing" dose. The percentage of the retained activity that could be flushed out fell to 68.5 per cent after 24 hours and to 47.2 per cent after 3 days. But even in the latter case 65 per cent of the activity was free folic acid. It seems clear from these experiments that folic acid is initially removed from the circulation and accumulated in the cells as such but is then relatively slowly converted into a nondisplaceable form at a rate that is dependent on the dose of folic acid administered.

Disposal of p-aminobenzoylglutamate. In view of the rapid intracellular uptake of folic acid we thought it of interest to compare the behavior of tritium-labeled *p*-aminobenzoylglutamate, which differs from folic acid only in the absence of the pteridine structure. This substance, when given intravenously in a dosage of 7.62 µg per kg (equivalent on a molar basis to a folic acid dose of 15 µg per kg) was found (Figure 8) to distribute itself into a maximal volume of 220 ml per kg, corresponding approximately to the plasma and interstitial water spaces. Urinary excretion was rapid, the clearance being 360 ml per minute, suggesting active tubular excretion. A total of 94 per cent of the dose was recovered from the urine in 24 hours. It appears, therefore, that the pteridine moiety of the molecule is essential for active uptake into the cells.

Distribution of urinary radioactivity. Folic acid isolation procedures suggested that part of the radioactivity present in the urine after an in-

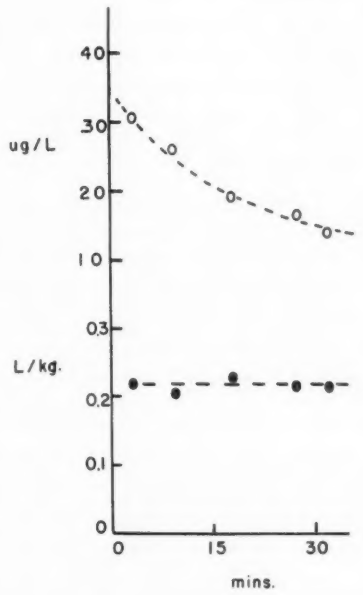

FIG. 8. INTRAVENOUS INJECTION OF 7.6 µG PER KG OF TRITIATED *p*-AMINOBENZOYLGLUTAMATE INTO A NORMAL SUBJECT. O----O, Plasma radioactivity; ●——●, volume of distribution of retained activity.

TABLE III

Distribution of urinary radioactivity before and after a flushing dose of unlabeled folic acid

	0.5–1 hour after initial dose*		0–1 hour after flushing dose		2–3 hours after flushing dose	
	Activity in urine	Excreted	Activity in urine	Excreted	Activity in urine	Excreted
	%	*dpm/hr*	%	*dpm/hr*	%	*dpm/hr*
Pteridine zone	22.2	14.6×10^4	1.9	7.6×10^4	2.2	5.8×10^4
p-Aminobenzoyl-glutamate zone	45.1	29.6×10^4	6.0	24.1×10^4	5.4	14.0×10^4
Folinic zone	26.7	17.5×10^4	1.3	5.2×10^4	1.4	3.6×10^4
Folic zone	3.5	2.3×10^4	90.1	361×10^4	89.6	232×10^4
Pteroyltri-glutamate zone	0.0	0.0×10^4	0.0	0.0×10^4	0.0	0.0×10^4
"NaOH" eluate	2.5	1.6×10^4	0.7	2.8×10^4	1.4	3.6×10^4
Total		65.8×10^4		401×10^4		259×10^4

* Folic acid containing 4.81×10^7 dpm.

travenous dose of tritium-labeled folic acid represented unchanged folic acid. This is in agreement with the finding of Anderson and colleagues (8) that much of the radioactive material excreted in the urine after an *oral* dose of tritiated folic acid is folic acid or its analogs.

In an attempt to identify some of the other radioactive compounds present, a dose of 1.5 μg per kg (22 μc) of tritium-labeled folic acid was administered intravenously to a normal adult male subject and a flushing dose of 30 mg of nonradioactive folic acid given at 6 hours; urines were collected both before and after the flushing dose and chromatographed after the addition of carriers:

One mg each of *p*-aminobenzoylglutamic acid, folinic acid, folic acid, and pteroyltriglutamic acid was dissolved in 1 ml of 0.2 per cent $NaHCO_3$. This solution was added to 10 ml of urine. The urine was diluted to a volume of 120 ml with distilled water and applied to a 1×7 cm column of DEAE-cellulose prepared and equilibrated as described in Methods. A flow rate of 0.4 ml per minute was used and the effluent collected after being passed through a recording ultraviolet absorptiometer. After application of the diluted urine, the column was developed with phosphate buffers of 0.01, 0.03, 0.06, 0.2, and 0.4 M, at pH 6.9. At the end of the run the column was cleared with 0.5 N NaOH solution. The location of the carriers in the collected fractions was determined from the absorptiometer record, identification being aided by determination of diazotizable amine in the various fractions before and after reduction by the Bratton-Marshall method. The distribution of radioactivity found is shown in Table III. All of the radioactivity applied to the columns was recovered in the six fractions listed.

It will be noted that in the second half-hour after injection, the rate of excretion of tritiated folic acid was very low, but there was a considerable excretion of *p*-aminobenzoylglutamate and pteridines. The latter were roughly in the proportions to be expected from the biological splitting of folic acid. Since no precautions were taken in these experiments to maintain a reducing milieu in the urine, at least some of these latter fractions were probably formed in the urine by the breakdown of reduced folates. No pteroyltriglutamate was detected in any of the samples. In the samples obtained after the flushing dose of folic acid there was a great increase in the rate of folic acid excretion but only such gradual decline in the excretion of the metabolites as might have been expected in the absence of flushing.

DISCUSSION

The avidity with which folic acid is removed from the plasma by the tissues is quite remarkable. In the experiments in which 1 μg per kg was injected, 90 to 95 per cent was removed in 3 minutes and 60 per cent in one circulation time. This suggests that a high affinity for folic acid must be a property of most tissues. This high affinity leads to a high relative concentration of folic acid inside cells. A similar rate of disappearance for folic acid has been reported by Chanarin, Mollin and Anderson (12) using a microbiologic assay method, and for folinic acid by Spray and Witts (15). On the other hand, Methotrexate (4-amino-

10-methylpteroylglutamic acid) (16) and *p*-aminobenzoylglutamic acid are not so treated and indeed appear to be restricted largely to the extracellular space. Two explanations can be offered for the tissue accumulation of folic acid: specific binding within the cells or a selective uptake process mediated by transport across the cell membrane or both. That the selective uptake mechanism is certainly operational is shown by the differences in behavior between folic acid and the other two compounds. The failure of Methotrexate and *p*-aminobenzoylglutamate to be accumulated in the tissues results from a failure to penetrate into the intracellular space. This defect is most readily explained if there is a specific membrane transport process for folic acid which is unable to transport the other two substances. The process of uptake is thus highly specific and is dependent not only on the presence of the pteridine ring but of specific substituents upon it. It would in any case be difficult to explain the rapid uptake of a molecule as bulky, polar, and lipoid-insoluble as folic acid without invoking a specific transport process. The accumulation of folic acid within the cells appears, therefore, to be due to a "pump and leak" mechanism similar to that described for amino acids and for cations (17). In this kind of process the substance is pumped into the cell but tends to diffuse back, and the steady state cellular concentration depends on the balance of these two processes. The very high intracellular concentration of folic acid observed points to an accumulative process with a high affinity and capacity. Whether a binding of folic acid to specific sites within the cell also occurs we are unable to say from the present data.

It has been estimated that the daily intake of folic acid in the diet is 100 to 200 μg (18); this amount could be transported to the storage sites without the blood level's ever exceeding 0.5 μg per L. Our results are entirely consistent with the view of Herbert (19) that there is little or no free folic acid in the fasting plasma and that the *L. casei* factor of plasma is some other form, perhaps tetrahydropteroyltriglutamate. However, the present data do not provide evidence of any precise level of free folic acid in plasma.

The gradual decrease with time in the amount of folic acid that can be flushed out of the cells can be explained either by a gradual redistribution of the folic acid into tissues that hold folic acid more tenaciously or, more likely, the conversion of free folic acid into tissue storage forms such as the "prefolic A" of Donaldson and Keresztesy (20).

The relatively small dependence of the urinary clearance of folic acid on the plasma concentration points up the unimportant role of the kidney in conservation of this vitamin. Renal conservation appears to be unnecesary in view of the high storage capacity of the tissues and consequent prerenal deviation of ingested folic acid. Our interpretation of the renal clearance data here differs radically from that given by Condit and Grob (21). These authors found a similar clearance but interpreted it as due to free glomerular clearance followed by proportionate tubular reabsorption. This assumption was based on their finding of very little binding of folic acid to plasma. We, on the other hand, find an average of 64 per cent of the plasma folic acid bound, irrespective of concentration. It is of interest in this connection to note that Freeman (16), using similar methods, found an average of 46 per cent of Methotrexate to be protein-bound. Condit and Grob, in their calculation of the clearance of endogenous folic acid, also do not consider the possibility that much of the plasma folic acid activity measured microbiologically may not be due to folic acid itself. Determinations with tritiated folic acid are free from this difficulty and indicate clearances of about 20 to 40 ml per minute with concentrations of 7 to 24 μg per L. This is the level of normal plasma folic acid activity found by Herbert and colleagues (22) by microbiological assay. Condit and Grob find a urinary clearance of less than 1 ml per minute for endogenous folic acid activity. The discrepancy in the clearance values found by the microbiological and radiochemical methods seems to be another indication that little of the endogenous plasma folic activity is actually folic acid.

The other compounds excreted in the urine are of some interest. Considerable amounts of labeled folinic acid appear to be excreted in the urine, but the concentration falls with time and does not seem to be increased by a flushing dose of folic acid. This probably indicates that folinic acid formation parallels the amount of free folic acid within the cells which is decreasing with time after the injection, and thus the rate of production

of folinic acid also decreases with time. After a flushing dose it might be expected that the excretion of *labeled* folinic acid would fall. This appears to occur. Similar changes occur in the pteridine and *p*-aminobenzoylglutamate fractions, which are probably derived from tetrahydropteroylglutamate. The lack of any activity in the pteroyltriglutamate fraction in the urine is perhaps not surprising since, if a pteroylpolyglutamate were flushed out of cells, it would probably be rapidly hydrolyzed by serum folic acid conjugase (23). The inability to demonstrate pteroyltriglutamate does not mean that *reduced* triglutamates are not excreted since, even if they were stable under these conditions, which is unlikely, the buffer concentrations needed for their elution from the column may be quite different from that required for pteroyltriglutamate (24).

It must be pointed out in this connection that identification of folic acid derivatives by the chromatographic procedures we have used are by no means rigorous. Materials are identified by their position in an elution system corresponding to known markers, but the possibility of coincident elution of other labeled compounds with similar chromatographic properties has not been excluded for substances other than folic acid itself. Chromatography in several systems to a constant specific activity would be required but has not yet been attempted.

SUMMARY

1. Tritium-labeled folic acid was administered intravenously in man at dosage levels of 1, 15, 150, and 1,430 μg per kg. Blood levels were followed for several hours. The fraction taken up by the tissues was greater at the lower dosage levels.

2. Tritium-labeled folic acid could be displaced from the cells by large flushing doses of unlabeled folic acid at any time up to 3 days after the initial dose. The amount that could be displaced decreased with time.

3. At low dosage levels most of the urinary radioactivity consisted of metabolic products of folic acid. At high dosage levels or after a flushing dose of unlabeled folic acid, as much as 90 per cent of the urinary radioactivity was found to be due to unchanged folic acid.

4. Tritium-labeled folic acid in plasma was 64 per cent protein-bound, the renal clearance at plasma levels above 5 μg per L averaging 51 ml per minute. At very low plasma levels the renal clearance fell.

5. The uptake of folic acid appeared to be a specific process dependent both on the pteridine structure and on substituents on the pteridine ring.

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THE METABOLISM OF NORMAL PLASMA PROTEINS AND GAMMA-MYELOMA PROTEIN IN MICE BEARING PLASMA-CELL TUMORS

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Study of the synthesis, catabolism and nature of γ-globulin has been facilitated by the availability in mice of transplantable plasma-cell tumors, each capable of synthesizing characteristic and different forms of plasma globulin (1). Since the use of such tumors in inbred mice readily permits multiple comparable observations, a study was carried out in sufficient detail to permit evaluation of the effects of different plasma-cell tumors on the metabolism of individual serum proteins. The findings were compared with those in human multiple myeloma in which published results are few and apparently conflicting. Thus, Berson and Yalow (2) found the fractional turnover rate of a γ-myeloma protein to be approximately normal in the donor patient as well as in another patient without a serum myeloma protein. Lippincott and coworkers (3, 4), however, observed a rapid turnover of normal γ-globulins and γ-myeloma proteins in patients with γ-myeloma proteins. Gammaglobulin turnover was less rapid in patients with β-myeloma proteins. Neufeld (5) also noted a rapid turnover for globulins in a patient with a γ-myeloma protein.

Two tumors were selected for the present study. The plasma-cell tumor 5563 (6) produces a γ-myeloma protein that is a γ-globulin on immunoelectrophoresis and a single 6.5S component on ultracentrifugal analysis. This is analagous to the γ-type myeloma proteins in man. A second mouse plasma-cell tumor, 5647 (7), however, produces a β-myeloma protein with ultracentrifugal components of 9, 11 and 13S and distinctive immunological properties similar to the characteristic features of beta (β_{2A})-type myeloma globulins in man. The serum levels of the normal γ-globulin components are reduced with the 5563 tumor, but this is less evident with the 5647 tumor.

Purified albumin, normal γ-globulin, and 5563 γ-myeloma protein from the mouse and normal human 6.6S γ-globulin were labeled with radioiodine. The metabolic behavior of these proteins was studied in normal mice as well as in mice bearing the γ (5563) plasma-cell tumor, the β (5647) plasma-cell tumor or a mammary carcinoma. This last tumor produced no serum myeloma protein but was transplanted and grew in the same way as did the others and thus served as a control for the effect of the presence of neoplasm per se on the turnover of these proteins.

The γ (5563) plasma-cell tumor has a profound effect, markedly increasing the turnover of both normal mouse γ-globulin and 5563 γ-myeloma globulin. Less marked effects were noted with the β (5647) plasma-cell tumor. Albumin turnover, however, was not altered by the tumors.

MATERIALS AND METHODS

Mice. C_3H/He and BAlb/c strains were obtained through the kindness of Dr. Michael Potter, National Cancer Institute, NIH, and the lines maintained by brother-sister mating.

Tumors. Plasma-cell tumor lines 5563 and 5647, both of which grow in C_3H mice, were obtained from Dr. Potter. The mammary carcinoma of C_3H mice was obtained from Dr. J. Craigie, and is maintained at the Imperial Cancer Research Fund Laboratories, Mill Hill. All three tumors are passed by subcutaneous implantation with a trochar, and grow during the course of 8 to 12 weeks to form large nodules, weighing up to 8 g before metastasis occurs. Tumor-bearing mice are in good general health until the terminal stage and show no renal abnormality. The paper electrophoretic patterns of serum obtained from mice bearing these tumors are illustrated in Figure 1.

Each plasma-cell tumor produces a characteristic protein: the 5563 tumor forms a large amount of γ-myeloma protein, while the 5647 tumor forms large amounts of β-myeloma globulin. The physicochemical and immunochemical properties of these myeloma proteins are described in detail elsewhere (8, 9). The mammary carcinoma was not associated with any significant changes

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in plasma proteins when examined by paper electrophoresis (Figure 1).

Proteins. Normal mouse γ-globulin was prepared from the pooled serum of normal adult C_3H mice or from pooled peritoneal fluid obtained from mice which had been hyperimmunized with hemocyanin (*Maia squinado*) in Freund's adjuvant (10, 11). Zone electrophoresis was carried out on polyvinyl chloride particle blocks (Geon 426) (12), and serial fractions from the γ-globulin region of each block were individually tested for the presence of γ-globulin and other serum proteins by the Ouchterlony technique (agar double-diffusion) employing potent rabbit antisera against normal mouse serum proteins and against normal mouse γ-globulin (9). Those γ-globulin fractions which were free from contaminating protein were pooled and concentrated by ultrafiltration (13), dialyzed against pH 7.4 sodium phosphate-buffered saline and tested for purity by starch gel electrophoresis and immunoelectrophoresis employing rabbit antinormal mouse serum. At no time in the preparation of the normal mouse γ-globulin or the 5563 myeloma protein were the fractions frozen.

The 5563 myeloma protein was prepared by the method of Askonas (14). Chromatography of serum containing approximately 7 g myeloma protein per 100 ml on columns of diethylaminoethyl (DEAE) cellulose was done (15), employing a gradient elution system (12), with an initial concentration of 0.015 M phosphate in a pH 6.5 potassium buffer and a limit concentration of 0.050 M phosphate while retaining pH 6.5. The central portion of the myeloma protein elution peak was pooled, concentrated, dialyzed and tested in the same manner as the normal serum γ-globulin.

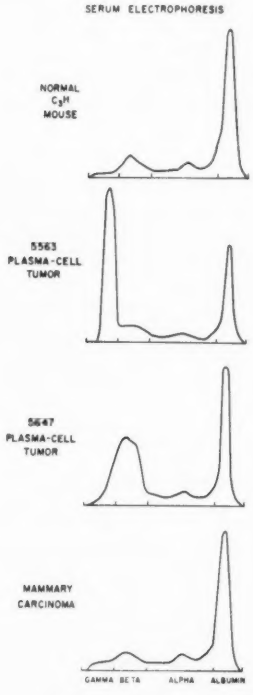

FIG. 1. SERUM ELECTROPHORETIC PATTERNS OF MICE. Zone electrophoresis under standard conditions (cf Methods) was carried out on paper strips stained by lissamine green and the protein distribution determined by densitometric tracing.

Mouse albumin was prepared from normal C_3H mouse serum by Geon block electrophoresis, and selection of the midportion of the albumin zone for concentration and dialysis against buffered saline as described above. The final preparation contained small amounts of a prealbumin and a postalbumin component when tested by starch gel electrophoresis.

Human γ-globulin was prepared by DEAE-cellulose chromatography of serum from a normal adult male donor. A gradient elution system, starting with a 0.01 M phosphate, pH 8 buffer, permitted separation of an initial fraction containing only 6.6S γ-globulins (12). No other fractionation procedure (i.e., ethanol) was employed. The starch gel electrophoretic properties of the serum protein fractions are shown in Figure 2.

Iodination with I^{131}. Iodination was performed by the iodine monochloride method of McFarlane (16); preoxidation with I^{127} at pH 4.6 was performed in the case of mouse albumin only. The amount of iodine introduced corresponded to 0.2 to 0.5 atom per mole of protein, taken as 160,000 mol wt for normal mouse γ-globulin, 5563 myeloma protein and normal human γ-globulin, and as 60,000 mol wt for mouse albumin. About 60 to 80 per cent of the added iodine became attached to protein. After iodination, free iodine was removed by passage through a column of Deacidite-FF in the chloride form (Permutit Co., Ltd., Gunnersbury, England); normal mouse serum was added so that the total radioactivity was less than 10 μc per mg protein, and the mixture was concentrated to a convenient volume by dialysis under pressure against physiological saline buffered at pH 7.0. The iodinated proteins, mixed with normal mouse serum, were tested by paper electrophoresis and found to have the characteristic electrophoretic mobilities of the parent proteins.

Estimation of radioactivity. Each mouse was housed in a separate cage, and was provided with drinking water containing 0.45 per cent NaCl and 0.01 per cent KI in order to accelerate renal excretion of free iodine and to reduce uptake of I^{131} by the thyroid gland to a minimum. Whole-body radioactivity was measured daily or more frequently, in the ring-counter described by Campbell, Cuthbertson, Matthews and McFarlane (17). The whole-body radioactivity was considered to be a good estimate of the retained labeled protein, for 98 to 99.5 per cent of radioactivity in numerous blood samples taken at various times during the tests was present as protein. Radioactivity in plasma samples was measured in a well-type scintillation counter, both before and after precipitation of protein. Protein was precipitated from blood or plasma samples with 10 per cent, wt/vol, trichloroacetic

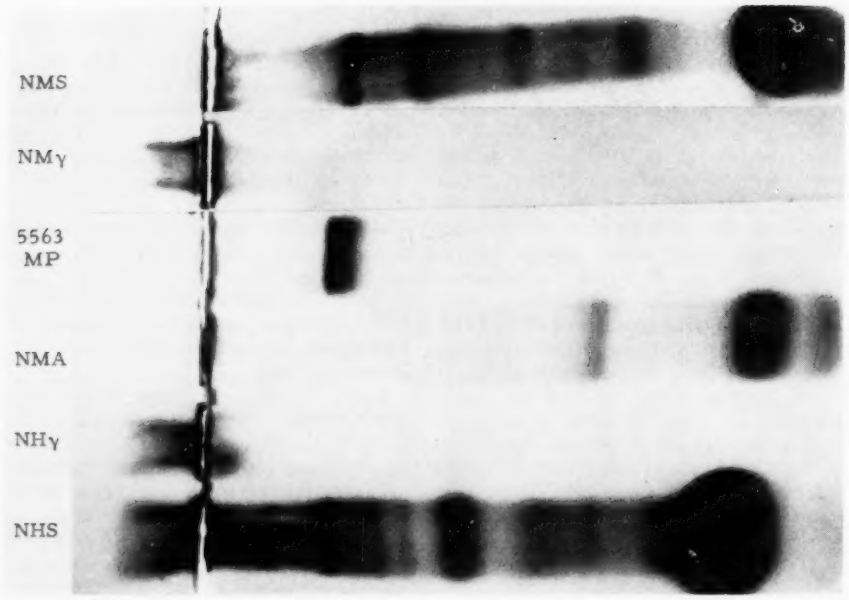

FIG. 2. STARCH GEL ELECTROPHORESIS OF NORMAL MOUSE SERUM AND SERUM PROTEIN FRACTIONS. Vertical electrophoresis was carried out in 0.03 M Tris borate buffer, pH 9.2, with a discontinuous buffer system (26, 27). NMS = normal mouse serum, NMγ = normal mouse γ-globulin, 5563 MP = myeloma protein from sera of mice bearing the 5563 plasma-cell tumor, NMA = normal mouse albumin, NHγ = normal human 6.6S γ-globulin, NHS = normal human serum.

acid. Counting was continued long enough to give an accuracy of at least ± 3 per cent. All figures were corrected for radioactive decay.

Blood samples during the experiments were taken from the retro-orbital venous plexus under ether anesthesia by a technique similar to that described by Halpern and Pacaud (18). At the end of the experiment the mice were sacrificed and bled out from the heart with a heparinized syringe. The terminal blood samples were used for determination of the hematocrit values. The mice were then weighed (allowance being made for the blood removed) and tumors were excised and weighed separately.

Methods of calculation. Blood volumes were calculated from the radioactivity of blood samples taken 2 to 3 minutes after injection of known amounts of radioactive protein into a tail vein. Plasma volumes were obtained from a knowledge of the blood volumes and the hematocrit values. The ratio of the extravascular to the intravascular plasma protein pools was calculated from the decay curve of plasma protein radioactivity as indicated by Wasserman and Mayerson (19). Approximately 15 μc (1.5 mg) of each material to be examined was injected into the tail veins of 4 or more mice. The findings within each group agreed closely except when there was a gross difference in tumor sizes. For this reason mean values are recorded, unless there was a reason for recording values individually for each mouse in a group. The half-time was determined from graphic plots of the total body radioactivity corrected for physical decay. The per cent I^{131}-protein degraded per day was obtained by dividing the observed half-time into 0.693, the natural logarithm of one-half. The quantity of any protein in the circulation (total circulating component), the total amount of exchangeable component, and the quantity of the protein component degraded per day were calculated in the usual way (17).

RESULTS

Turnover in normal mice

Mouse albumin. Approximately 15 μc (1.5 mg) I^{131}-mouse albumin obtained from C_3H mice was injected into four normal C_3H mice weighing 21 to 29 g (mean 27 g). The findings in respect to disappearance of radioactivity from the whole body are illustrated in Figure 3 where labeled albumin was found to have a mean half-life of 1.54 days. Since the plasma radioactivty was 98 to 99 per cent protein-bound at all times in the experiment, the whole-body radioactivity curves can be taken to represent I^{131}-protein catabolism.

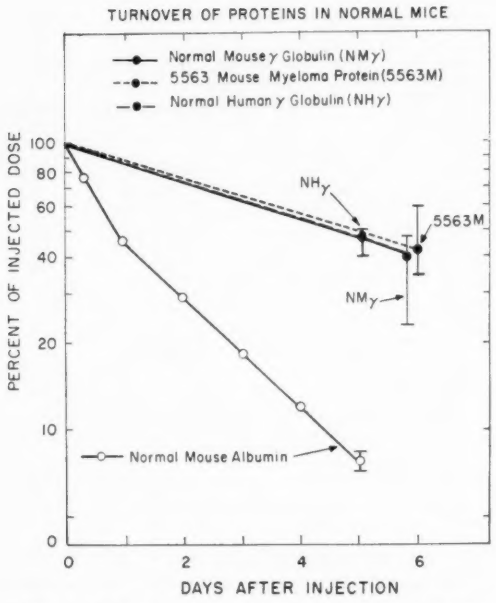

FIG. 3. MEAN ELIMINATION OF I^{131}-PROTEINS INJECTED INTO NORMAL MICE. The range of values indicated by the brackets includes observations in 11 mice (C_3H or BAlb/c) for normal mouse γ-globulin, 6 mice for human γ-globulin before immune response, 9 mice for 5563 myeloma protein and 4 mice for normal mouse albumin. Total body radioactivity counts were measured daily, corrected for physical decay and recorded on the ordinate as the per cent of injected dose. Daily mean values fell on the lines and are not shown unless the catabolic curves were not straight lines.

Normal γ-globulin. I^{131}-labeled normal C_3H mouse γ-globulins were administered to a total of eight normal C_3H mice in two separate experiments and were found to have a mean half-time of 4.6 days, as shown in Figure 3. The same half-time was observed when γ-globulin was given to normal BAlb/c mice. In order to check that the half-life of the various proteins obtained by whole-body radioactivity was close to their half-life in the plasma, blood samples were examined daily in several tests. Figure 4 shows that the elimination rate for normal mouse γ-globulin agreed well with that observed by whole-body counting. The mean value for the half-life of normal mouse γ-globulin observed here is considerably longer than previously reported values (20). Reasons for this are considered in the Discussion.

5563 Myeloma protein. The 5563 γ-myeloma protein was treated by the normal mouse in the same manner as normal mouse γ-globulin. When injected into eight normal mice the mean was found to be 4.9 days (Figure 3). There was no difference in the behavior of 5563 myeloma protein when given to normal C_3H or BAlb/c mice.

Normal human γ-globulin. Approximately 1.5 mg of I^{131} human 6.6S γ-globulin was injected intravenously into five normal C_3H mice. Since no antibody formed during the first 5 days, it was possible to measure the elimination rate with reasonable accuracy before immune elimination occurred. Only whole-body counts were made in this experiment. It is evident from Figure 3 that normal mice treat human γ-globulin and mouse γ-globulin in a similar way, both having a half-life of 4.6 days.

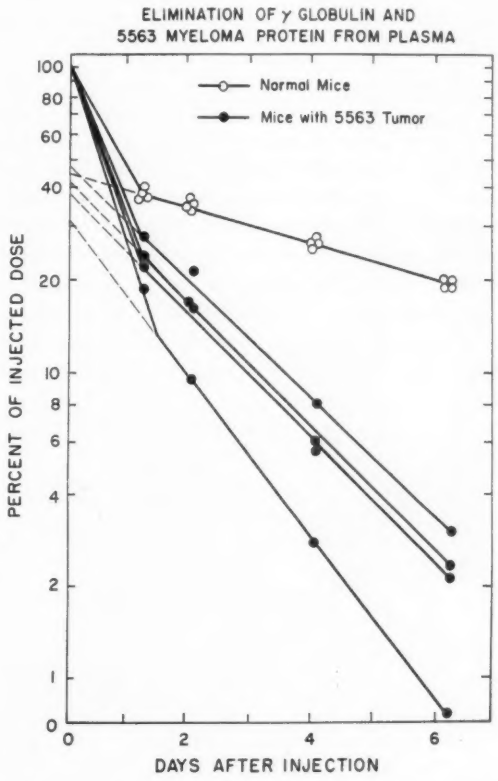

FIG. 4. ELIMINATION OF NORMAL MOUSE γ-GLOBULINS AND 5563 MYELOMA PROTEIN FROM THE PLASMA OF 4 NORMAL MICE AND 4 MICE BEARING THE 5563 PLASMA-CELL TUMOR (2 MICE OF EACH GROUP FOR ONE PROTEIN). The fraction of the initial isotope concentration in the plasma is recorded on the ordinate.

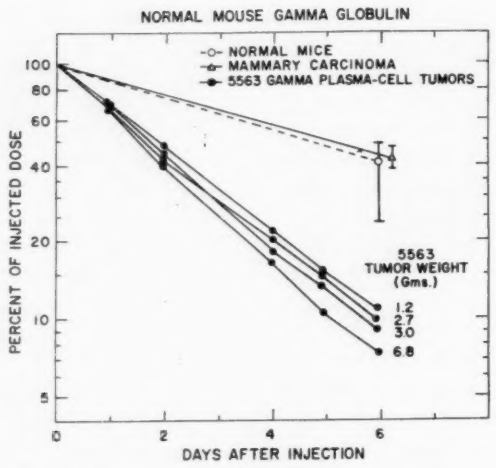

FIG. 5. ELIMINATION OF NORMAL MOUSE γ-GLOBULIN FROM TUMOR-BEARING MICE. The median weight of the mammary carcinoma tumors in 4 mice was 3.3 g.

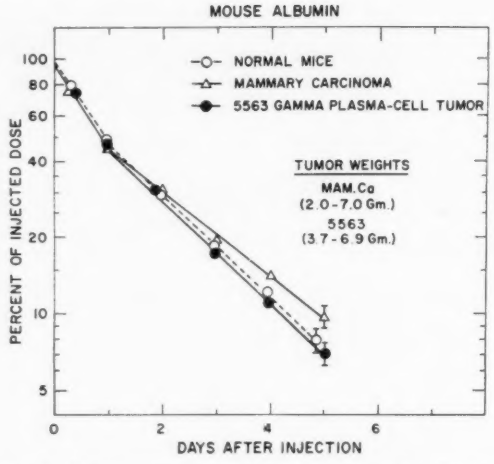

FIG. 7. ELIMINATION OF MOUSE ALBUMIN FROM TUMOR-BEARING MICE.

Turnover in tumor-bearing mice

5563 Plasma-cell tumor. This tumor, which produces a γ-myeloma protein (Figure 1), caused a striking increase in the turnover of normal mouse γ-globulin and 5563 myeloma protein (Figures 5 and 6) but no evident alteration in albumin turnover (Figure 7). Normal γ-globulin turnover was measured in four mice which bore tumors with an average weight of 3.4 g. The mean half-time was 1.8 days, compared with 4.6 days in normal mice. Even a 1.2 g tumor, which represented less than 5 per cent of the total body weight, had a profound effect on γ-globulin turnover (Figure 5).

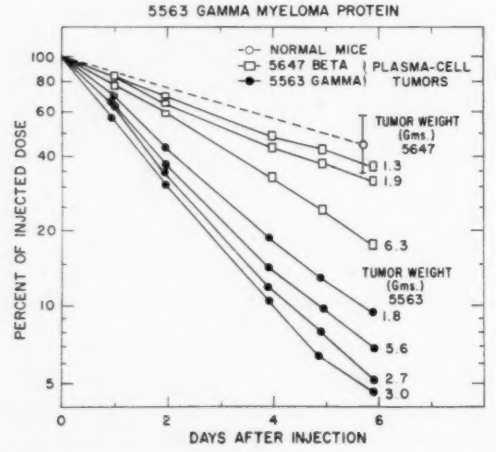

FIG. 6. ELIMINATION OF 5563 γ-MYELOMA PROTEIN FROM TUMOR-BEARING MICE.

The turnover of 5563 γ-myeloma protein was also markedly increased by the tumor. A mean half-time of 1.5 days was observed in four mice bearing tumors with an average weight of 3.3 g. The half-life of the various proteins obtained by whole-body counting was checked by measurement of their half-life in the plasma. The data in Figure 4, showing the elimination of I^{131}-labeled normal mouse γ-globulin, or 5563 myeloma protein (two in each group of four normal mice and four mice with 5563 plasma-cell tumor), correlate well with the elimination rates determined by whole-body counting.

Albumin turnover, by contrast, was not appreciably altered in four mice bearing 5563 tumors weighing 3.7 to 6.9 g (mean 4.9). As is shown in Figure 7, the half-time was 1.46 days.

Human γ-globulin turnover was also measured in four mice with 5563 tumors, as is shown in Figure 8, and was found to be accelerated (mean half-time 2.8 days) in mice with tumors weighing 3.7 to 6.9 g. This effect, although real, is less marked than that seen with the homologous globulins.

5647 Plasma-cell tumor. This β-globulin-producing tumor had less effect on γ-globulin turnover than did the γ-type plasma-cell tumor (Fig-

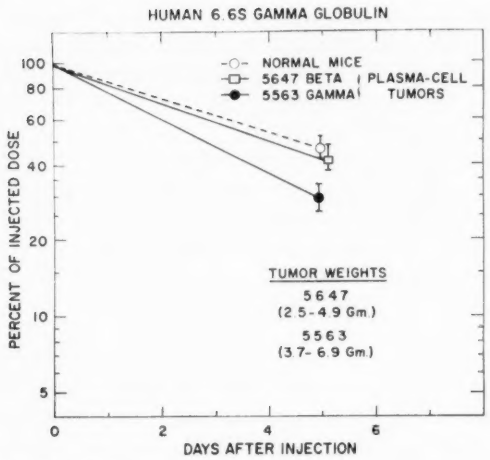

FIG. 8. ELIMINATION OF HUMAN 6.6S γ-GLOBULIN FROM TUMOR-BEARING MICE.

ure 6). The 5563 γ-myeloma protein had a mean half-time of 3.8 days in five mice bearing 5647 tumors weighing 1.3 to 6.3 g (mean 3.2), in contrast to a half-time of 4.9 days in normal mice and to 1.5 days in mice with 5563 tumors of similar size.

Human γ-globulin turnover was also less markedly affected, the mean half-time being 4.1 days for seven mice with 5647 tumors (Figure 8), compared with 4.6 days in normal mice and 2.8 days in mice with the 5563 tumor. Albumin turnover was not measured.

Mammary carcinoma. This tumor grew at about the same rate as did the plasma-cell tumors but caused no evident change in the plasma proteins. It served as a control for the effects of the presence of malignant tissue per se on plasma protein metabolism. As is shown in Figure 5, the turnover of mouse γ-globulin was normal, the mean half-time being 4.8 days in four mice with a mean tumor weight of 3.3 g. Albumin turnover was slightly prolonged, with a mean half-time of 1.9 days in four mice whose tumors weighed 2 to 7 g (mean 5; Figure 7).

Rates of protein synthesis

The intravascular pool of serum protein, determined from the measurements given in Figure 4, constituted about 45 per cent of the total in the body. In Table I are summarized the values obtained for blood and plasma volume and for the ratio of intravascular to total plasma protein.

Albumin synthetic rate was calculated from the data assembled in Table II. Approximately 116 mg of albumin is synthesized per 100 g of mouse weight—i.e., about 29 mg albumin per day for each 25 g normal mouse. The synthetic rate and the fractional rate of albumin degradation were not markedly altered by the presence of the 5563 plasma-cell tumor. The serum albumin concentration was substantially reduced, however, by a dilutional effect from increased plasma volume and increased extravascular distribution of albumin.

Normal γ-globulin synthesis was only one-twentieth that of albumin. As is seen in Table II, approximately 5.2 mg of γ-globulin was synthesized per 100 g mouse weight, or 1.3 mg per 25 g mouse.

Presence of the 5563 plasma-cell tumor more than doubled the fractional rate of normal γ-globulin removal. Calculation of the rate of normal

TABLE I

Effect of tumors on volume of protein distribution

Group	No.	Range of tumor weights	Blood volume	Plasma volume	Approx. ratio, intravasc. and total plasma protein
		% total body wt	*ml/100 g total wt*	*ml/100 g total wt*	
Normal C_3H	2		7.0–7.25	3.9	1:2.2
Normal BAlb/c	5		6.5–7.5	3.2–3.8	1:2.2
5563 Plasma-cell tumor	4	4–10	6.7–7.6	4.2–5.0	1:2.2–2.7 (2.5)
5647 Plasma-cell tumor	1	20	9.3	5.9	1:2.0
Mammary carcinoma	4	6–20	9.6–11.2	6.1–7.1	not done

TABLE II

Comparison of body content, turnover, and synthetic rate of plasma proteins in normal and tumor-bearing mice *

	Normal γ-globulin					5563 γ-myeloma protein		Albumin		
	Normal mouse	5563 Tumor		5647 Tumor		Mammary carcinoma	5563 Tumor	Normal mouse	5563 Tumor	Mammary carcinoma
Concentration in serum (*g/100 ml*)	0.40	if 0.20†	if 0.10†	if 0.40†	if 0.20†	0.50	4.8	3.0	2.0	2.8
$T_{\frac{1}{2}}$	4.6	1.8		3.8†		4.8	1.5	1.54	1.46	1.9
Plasma volume (*ml/100 g mouse wt*)	3.9	4.6		5.9		6.6	4.6	3.9	4.6	6.6
Ratio total exchangeable: intravascular protein	2.2	2.5		2.0		2.2†	2.5	2.2	2.5	2.2†
Total circulating component (*mg/100 g mouse wt*)	15.6	9.2	4.6	23.6	11.8	33.0	221.0	117	92	185
Total exchangeable component (*mg/100 g mouse wt*)	34.3	23.0	11.5	47.2	23.6	72.6	553.0	257	230	406
% I^{131}-protein degraded/day‡	15	38.5		18.2		14.4	46.2	45.0	46.3	36.4
Amount degraded amount synthesized (*mg/100 g/day*)	5.2	8.9	4.4	8.6	4.3	10.5	256.0	116	107	148

* Calculated on basis of mean tumor weights of 11.2 g (5563 tumor), 10.6 g (5647 tumor), 10.9 g (mammary carcinoma) and liver weight of 6 g per 100 g mouse weight.

† Values based on estimations described in the text.

‡ % I^{131}-protein degraded per day $= 100\left(1 - \frac{0.693}{T_{\frac{1}{2}}}\right)$.

γ-globulin synthesis in the presence of this tumor, however, requires an assumption as to the level of the normal γ-globulin components. The large amount of myeloma protein overshadows much of the γ-globulin region and prevents quantitative measurement of the normal components, but immunoelectrophoresis and starch gel electrophoresis have shown that normal γ-globulin components are reduced (8, 9). Since such analyses were not susceptible to quantitative assessment, two sets of calculations are made in Table II, based on the assumption that normal γ-globulin concentrations were in the range of one-half to one-quarter the normal level. If these assumptions are correct, the rate of γ-globulin synthesis was about normal in the presence of the 5563 tumor.

The β-globulin-producing 5647 plasma-cell tumor had much less effect on the fractional rate of γ-globulin removal than the γ-globulin-producing 5563 tumor, although the mean tumor weights were approximately the same: 3.2 and 3.4 g, respectively.[1] Difficulties in measuring the level of normal γ-globulins were encountered also with the 5647 tumor, but qualitative tests indicated that the γ-globulins were not so markedly reduced with this tumor and, therefore, calculations were made, assuming normal or half-normal γ-globulin levels. Gamma-globulin synthesis, as shown in Table II, appeared to be normal in the presence of this tumor.

Gamma-globulin levels were slightly elevated by the presence of the mammary carcinoma, perhaps because some of the tumors were partially necrotic. The fractional rate of γ-globulin removal was normal with these tumors (Table II), although the rate of γ-globulin synthesis appeared to be increased.

The 5563 myeloma protein was very rapidly degraded in the presence of the 5563 tumor, almost half of the protein being removed and replaced per day. The rate of 5563 myeloma globulin synthesis was calculated to be approximately 19 mg protein per g tumor per day.

DISCUSSION

The catabolic behavior of γ-globulin differed notably from that of albumin in the mouse. The half-life of 4.6 and 1.5 days for these two proteins differs considerably and, in this feature, stands apart from the usual finding in species other than

[1] The calculations of the catabolism of normal mouse γ-globulin in the mice bearing the 5647 tumor are based on the turnover of γ-myeloma protein administered to these mice. Because of the similarity of normal γ-globulin and 5563 γ-myeloma protein metabolism in normal and 5563 tumor-bearing mice, it seems reasonable to believe that this will apply also for mice with the 5647 tumor.

rodents (20). The mean half-life of 4.6 days for normal mouse γ-globulin is longer than the 1.9 days previously reported (20). The reason for this may be that the materials which we used were prepared and iodinated by techniques that more effectively avoided denaturation, or that the γ-globulin used in previous reports contained other serum globulins, since uncontaminated γ-globulins are difficult to obtain from mouse serum. In this connection it is worth noting that the γ-globulins tested in the present study had antibody activity and were isolated by preparative electrophoresis with care, in order to obtain only uncontaminated γ-globulin fractions. The γ-globulins were not frozen after purification, and iodination was carried out with a molecular ratio for iodine: γ-globulin of 1 : 2 or less.

The factors controlling catabolism of normal γ-globulins and other serum proteins are unknown. Campbell and co-workers (17) studied the behavior of separately labeled albumin and γ-globulin in the rabbit and rat and concluded, because of differences in urinary and plasma specific activity curves, that these two proteins were metabolized by independent processes. This view has been confirmed in the present studies where γ-globulin catabolism was accelerated by a plasma-cell tumor without any effect on albumin catabolism.

Differences in the metabolic effects of individual lines of malignant plasma cells were clearly evident. The β-globulin-producing 5647 mouse tumor had a less marked effect than had the 5563 tumor. This difference in effect between the two plasma-cell tumors is in agreement with the observations of Lippincott and colleagues (3) that normal γ-globulin turnover was greater in patients with γ-myeloma proteins than in those with β-myeloma proteins.

Inasmuch as catabolism of plasma proteins occurs predominantly or exclusively in close association with the blood stream (17, 21) a possible explanation for the differences observed might be available if the ratio of intra- to extravascular protein were much increased in mice with 5563 tumors. This was not found to be the case. Another possibility would be that γ-globulins were being lost through the glomeruli, and broken down during reabsorption by the renal tubules; but mice bearing the 5563 tumor do not show renal abnormalities or increased proteinuria. The remaining alternatives appear to be that either the presence of 5563 γ-myeloma protein as such, or the presence of the tumor, is responsible in some other way.

It is not clear, however, that a simple gross increase in γ-globulin level is entirely responsible for the increased rate of γ-globulin destruction. Although, in general, mice with the highest levels of 5563 myeloma protein had the highest γ-globulin elimination rates, the correlation was not complete nor was there any simple relationship between the two. Even the mice with a 1.2 g tumor showed elimination rates 2.1 times normal, while those with the largest neoplasm (6 g) were 2.9 times normal. Sufficient 5563 myeloma protein was not available to test directly the effect of maintaining high plasma levels of this protein in the absence of a neoplasm. It seems unlikely that the presence of the protein is the entire explanation, since Humphrey and McFarlane (22) found that rabbits, with γ-globulin increased to 4 times normal levels by hyperimmunization, had the same fractional rate of γ-globulin removal as had normal rabbits. The mouse with a 1.2 g 5563 plasma-cell tumor had a comparable increase of his total γ-globulins but the rate of γ-globulin removal was markedly increased.

It is interesting that the catabolic behavior of the 5563 γ-myeloma protein and of normal mouse γ-globulin was similar in all groups of mice in which comparison was made, although the finding is perhaps not surprising in view of the physicochemical and immunological resemblance of the proteins. More unexpected is the similarity in rate of removal and catabolism of human and rabbit (20) γ-globulin to mouse γ-globulins, their half-life in mice being almost the same. Whatever the factors that determine their catabolic rate, it is apparent that there are some relevant differences between mouse and human γ-globulin molecules, since the presence of the 5563 plasma-cell tumor caused a greater increase in the rate of turnover of the one than of the other.

In view of the evidence that myeloma proteins in both mouse and man can be classified into two major groups, the γ-type and β (or β_{2A})-type myeloma proteins (8), and because of the physicochemical and immunochemical differences between these groups, a comparison of the metabolic

behavior of representative proteins from these two groups would be important. Although it was not possible to carry out a thorough comparison, in the course of the present work preliminary studies were undertaken with a β-type myeloma protein associated with the MPC-2 plasma-cell tumor of BAlb/c mice (23). The half-life in normal mice of this β-myeloma protein was much shorter (about 1 day) than that noted above for a γ-myeloma globulin. In the case of the MPC-2 myeloma protein, however, some denaturation had almost certainly occurred in preparation, and the true value may be somewhat greater. The MPC-2 tumor also produces a urinary Bence Jones protein (23). When the Bence Jones protein was labeled and injected intravenously, plasma and whole-body radioactivity fell very rapidly, virtually all having been eliminated in 24 hours. Approximately 30 per cent of the radioactivity in the urine was bound to protein, presumably in the form of unchanged Bence Jones protein.

The 5563 myeloma protein was synthesized *in vivo* at the rate of 19 mg protein per g (wet weight) of plasma-cell tumor per day. This figure determined by I^{131} turnover studies may be compared with the value of 14 mg per g tumor per day estimated in studies of the turnover of protein labeled with C^{14}-lysine *in vivo* (24). Askonas (14), however, has shown that 5563 tumor slices form protein at a slower rate *in vitro*—i.e., 1.5 to 2 mg per g tumor per day, approximately one-tenth the *in vivo* rate.

Synthesis of 5563 myeloma protein occurs as rapidly in the plasma-cell tumor as does synthesis of albumin in mouse liver, which also forms approximately 19 mg albumin per g tissue each day.

Low serum albumin levels, a common feature of malignant disease in man, were found in the animals bearing the tumors used in the present study. Steinfeld (25) studied the turnover of albumin in 12 patients with neoplastic disease and found that the percentages of body albumin degraded and synthesized per day were abnormal except in the one patient with multiple myeloma. Neufeld (5) reported a normal half-time for albumin in two patients with myeloma. Similarly in mice, the mammary carcinoma depressed the percentage of albumin degraded and synthesized per day, although the 5563 mouse plasma-cell tumor did not have this effect. Speculation on the significance of a difference between the effects of carcinomatous and plasma-cell malignancies on albumin metabolism, however, should await further study.

SUMMARY

Transplantable plasma-cell tumors in mice closely resemble multiple myeloma in man, and both result in decreased serum levels of normal γ-components and of albumin, in the presence of large amounts of myeloma protein.

The metabolism of normal mouse γ-globulin, of a mouse γ-myeloma protein, of normal human γ-globulin, and of mouse albumin was studied in normal mice or tumor-bearing mice by means of purified proteins labeled with I^{131}.

The tumors were a γ-type (5563) plasma-cell tumor, a β-type (5647) plasma-cell tumor, and a mammary carcinoma with similar growth characteristics.

In normal mice the half-time for the turnover of all three γ-globulins was 4.6 days, while the half-time for albumin was 1.54 days.

The γ-type plasma-cell tumor caused a very marked acceleration of γ-globulin catabolism, while the β-type tumor had much less, and the mammary carcinoma no such effect. Neither in normal nor in tumor-bearing mice was there any difference between the behavior of γ-myeloma globulin and of normal γ-globulin.

In normal mice about 20 times more albumin than normal γ-globulin was synthesized each day. The synthesis of normal γ-globulin and of albumin was unaltered by the presence of the plasma-cell tumor.

The 5563 plasma-cell tumor synthesized about 19 mg of myeloma protein per g wet weight per day. This rate is at least as great as that of albumin synthesis in the liver.

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THE PHYSIOLOGICAL SIGNIFICANCE OF THE SECRETION OF ENDOGENOUS INSULIN INTO THE PORTAL CIRCULATION. IV. HEPATIC UPTAKE OF GLUCOSE DURING GLUCOSE INFUSION IN NON-DIABETIC DOGS *

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Recent studies from this laboratory (1) have shown that insulin has an immediate and profound effect upon net hepatic glucose balance. When insulin was administered at a rate (2) estimated to minimize the counter-regulatory responses to hypoglycemia, the decline in blood glucose which ensued was largely the consequence of a diminished hepatic release of glucose (1).

Since, under physiologic circumstances, endogenous insulin secretion is stimulated by the rising blood glucose concentration (3, 4) which follows a carbohydrate meal, the effect of insulin in regulating hepatic glucose metabolism is best assessed in the presence of a glucose load. Whether or not hyperglycemia, with its attendant release of endogenous insulin, results in a decrease or cessation of hepatic glucose output has been a controversial subject (5–13). However, in all but one of the previous studies (5), the conclusions were based upon inferential data, since net hepatic glucose balance was not measured.

The present studies were undertaken to determine the effect of hyperglycemia and the concomitant release of endogenous insulin upon hepatic glucose metabolism. Hyperglycemia was produced by the intravenous infusion of glucose. The rate of glucose administration was varied in order to define better some of the factors that control hepatic glucose output. Dogs with complete end-to-side portacaval shunts were used, since this preparation separates the circulation of the liver from that of the remainder of the splanchnic bed and thereby permits measurement of hepatic rather than splanchnic glucose balance (1).

METHODS AND PROCEDURE

Twenty-two studies were performed in dogs in which end-to-side portacaval shunts had been performed at least 2 weeks earlier. The dogs were anesthetized with Nembutal (25 mg per kg i.v.) 15 hours after food had been removed from their cages. Previously they had been maintained on a ration in which approximately 50 per cent of the total calories was derived from carbohydrate, 20 to 30 per cent from protein, and the remainder from fat. Hepatic venous blood samples were collected through a cardiac catheter inserted deep into a hepatic vein under fluoroscopic control. Position of the catheter was checked at intervals during each experiment. Arterial blood samples were obtained through a Cournand needle placed in a femoral artery.

Hepatic blood flow and the concentration of glucose in arterial and hepatic venous blood were determined at 10-minute intervals throughout the study. Hepatic blood flow was estimated by the clearance-and-extraction method of Bradley, Ingelfinger, Bradley and Curry (14), with I^{131}-labeled rose bengal as the extractable material (15). Blood glucose was determined in triplicate on each blood sample by the Somogyi copper iodometric method (16,17). The methods used in this laboratory have been described in detail in a previous publication (1). Hepatic glucose balance in milligrams per minute at each 10-minute interval was calculated as the product of the estimated hepatic blood flow and the hepatic venous-femoral arterial glucose concentration difference.

Two to four measurements of hepatic glucose output were obtained during a control period. In 11 studies, changes in hepatic glucose balance were then followed during the administration of a constant infusion of glucose into a hind leg vein. In some studies, after the glucose was stopped, additional measurements were obtained during the "recovery" period.

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In 11 other studies the effects of insulin, administered prior to and during the glucose infusion, on hepatic glucose balance were determined. Glucagon-free insulin[1] was administered slowly by constant intravenous infusion for 30 to 60 minutes after control measurements had been made. Glucose was then infused, together with the insulin, for another 60 to 80 minutes.

RESULTS

1. Effect on hepatic glucose balance

A. Glucose infusion. In each of the 11 dogs glucose infusion produced a fall in hepatic glucose output (Tables I and II). Within 10 minutes mean hepatic glucose output fell from 50.7 to 27.6 mg per minute. This 46 per cent decrease in hepatic output of glucose was associated with a rise in mean arterial glucose concentration of only 9.4 mg per 100 ml—from 82.1 to 91.5 mg per 100 ml. By 50 minutes, mean hepatic glucose output fell to only 1.4 mg per minute, a 97 per cent change from the mean control value. From 60 to 90 minutes, hepatic glucose output ceased; instead, the liver was *extracting* an average of 20.2 mg per minute during this time (Table I). This represents a net conservation of glucose by the liver of 70.9 mg per minute.

A net uptake or positive balance of glucose across the liver was observed in 7 of the 11 dogs (Figure 1). In the other 4 animals, although net uptake of glucose by the liver did not occur, hepatic glucose output fell markedly during the infusion, averaging 5.3 mg per minute 90 minutes after glucose was started, a 91.2 per cent decrease from the mean control output of 60.5 mg per minute.

Inspection of the data (Table I) obtained from 11 dogs suggests that a positive glucose balance across the liver is related to the rate at which arterial glucose concentration increased. In the 7 dogs in which net hepatic uptake of glucose was observed, mean arterial glucose concentration increased 33 mg per 100 ml by 30 minutes and 65.5 by 90 minutes after starting glucose. By contrast, in the 4 dogs that did not attain positive hepatic glucose balance, arterial glucose concentration increased only 10 mg per 100 ml at 30 minutes and 32.2 at 90 minutes. In general, the more rapid rise in arterial glucose concentration in the "uptake" dogs was attributable to the infusion of larger glucose loads (Table I) and, more specifically, to the greater extent to which the infused loads exceeded the control hepatic glucose output. In considering the entire group of 11 animals, the quantity of glucose infused per minute was 3.5 to 5.8 times greater than the control hepatic glucose output during the 90 minutes of infusion in the "uptake" dogs, whereas it was only 0.9 to 2.4 times greater than control hepatic glucose output in the "non-uptake" dogs.

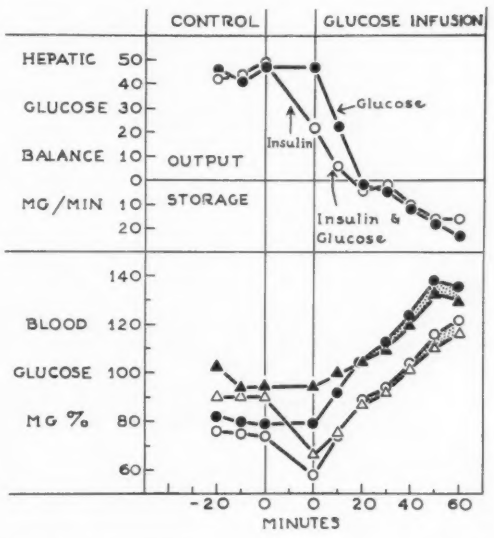

FIG. 1. COMPARISON OF MEAN CHANGES IN HEPATIC AND ARTERIAL GLUCOSE CONCENTRATIONS, AND HEPATIC GLUCOSE BALANCE IN DOGS DEMONSTRATING A NET HEPATIC UPTAKE OF GLUCOSE DURING GLUCOSE INFUSION ALONE AND DURING INSULIN AND GLUCOSE INFUSIONS. Solid symbols used for data in dogs receiving only glucose; open symbols for dogs receiving insulin and glucose infusions. For blood glucose data, triangles refer to glucose concentration in hepatic venous blood, circles to glucose concentration in arterial blood.

The mean arterial glucose concentration at which net uptake of glucose by the liver was first observed was 115.6 mg per 100 ml (Table III), a value 36.3 mg higher than the mean control. A rising arterial glucose concentration was not prerequisite for a positive hepatic glucose balance, since net uptake not only continued when arterial glucose concentration had reached a plateau, but also when arterial glucose concentration was falling (Table I, Dogs 522, 525, 610).

[1] We are indebted to Dr. W. R. Kirtley of the Eli Lilly Co. for the generous supply of glucagon-free insulin.

TABLE I

Effect of glucose infusion upon hepatic glucose balance *

Dog	Weight		Control values −30	−20	−10	0	Mean control	Time during and after glucose infusion 10	20	30	40	50	60	70	80	90	100	110	120
no.	*kg*																		
35	16.8	EHBF		178	176	188	181	177	163	207	187	174	187	183	179	175			
		HV		144.4	122.1	120.4	129.0	123.0	133.4	133.7	139.9	146.4	143.3	137.3	130.8	126.6			
		A		98.4	98.1	99.8	98.8	104.9	112.8	117.0	126.3	135.1	132.0	128.9	127.5	124.9			
		HV-A		46.0	24.0	20.6	30.2	18.1	20.6	16.7	13.6	11.3	11.3	8.4	3.3	1.7			
		HGB		81.9	42.2	38.7	54.3	32.0	33.6	34.6	25.4	19.7	21.1	15.4	5.9	3.0			
		Glucose						←———				50 mg/min				——→			
36	25.9	EHBF		160	187	262	203	229	191	237	164	183	165	181	198	211	213	231	229
		HV		116.5	137.6	112.0	122.0	108.6	110.3	117.0	120.4	120.7	121.0	130.0	133.1	138.7	126.9	112.5	104.1
		A		89.4	91.4	89.7	90.2	88.8	90.0	89.7	99.8	106.3	113.1	121.8	133.4	138.7	119.6	105.5	99.5
		HV-A		27.1	46.2	22.3	31.9	19.8	20.3	27.3	20.6	14.4	7.9	8.2	+0.3	0	7.3	7.0	4.6
		HGB		59.6	86.4	58.4	68.1	45.3	38.8	56.2	33.8	26.4	13.0	14.8	+0.6	0	15.5	16.2	10.5
		Glucose						←——50 mg/min——→			←——100 mg/min——→			←——150 mg/min——→			←——None——→		
319	17.2	EHBF		170	147	142	153	134	125	143	149	140	145	149	129	158	169		
		HV		120.7	117.4	111.9	116.7	108.3	112.2	110.2	113.5	111.9	112.7	109.1	110.8	109.1	101.4		
		A		85.5	84.9	83.8	84.7	82.5	84.7	84.4	92.1	96.5	99.0	100.1	100.9	99.2	93.7		
		HV-A		35.2	32.5	28.1	32.0	25.8	27.5	25.8	21.4	15.4	13.7	9.1	9.9	9.9	7.7		
		HGB		59.8	47.8	39.9	49.2	34.6	34.4	36.9	31.9	21.6	19.9	13.6	12.8	15.6	13.0		
		Glucose						←——50 mg/min——→			←——100 mg/min——→			←——150 mg/min——→					
513	19.0	EHBF	202	210	196	201	202	212	223	223	234	226	223	191	186	187	188		
		HV	111.8	111.8	108.5	102.1	108.6	101.8	99.4	100.2	100.4	101.3	113.4	121.8	122.6	117.7	106.7		
		A	75.6	74.8	74.8	69.9	73.8	84.5	92.6	96.4	98.0	99.1	117.2	121.2	121.0	113.7	104.0		
		HV-A	36.2	37.0	33.7	32.2	34.8	17.3	6.8	3.8	2.4	2.2	+3.8	0.6	1.6	4.0	2.7	5.1	
		HGB	73.1	77.7	66.1	64.7	70.4	36.7	15.2	8.5	5.6	5.0	+8.5	1.1	3.0	7.5	5.1		
		Glucose						←——100 mg/min——→					←——200 mg/min——→						
126	19.1	EHBF		200	206	200	202	208	205	277	250	329	298	372	366	298	309		
		HV		91.7	88.5	90.5	90.2	91.7	93.1	94.0	93.1	92.2	100.0	104.2	108.8	120.5	124.8		
		A		85.1	84.8	85.1	85.0	87.7	92.2	96.5	96.2	97.1	106.0	111.0	119.0	131.9	136.8		
		HV-A		6.6	3.7	5.4	5.2	4.0	0.9	+2.5	+3.1	+4.9	+6.0	+7.2	+10.2	+11.4	+12.0		
		HGB		13.2	7.6	10.8	10.5	8.3	1.9	+6.9	+7.8	+16.1	+17.9	+26.8	+37.3	+34.0	+37.1		
		Glucose						←——50 mg/min——→			←——100 mg/min——→			←——150 mg/min——→					
312	12.4	EHBF			442	463	453	419	379	410	399		402	428	342	369	403	267	267
		HV			74.8	69.3	72.1	76.6	71.5	72.1	80.2		97.4	99.7	108.6	113.9	101.3	80.5	71.3
		A			62.9	58.7	60.8	65.1	63.8	62.6	76.3		96.0	104.7	115.5	123.3	101.3	81.3	70.2
		HV-A			11.9	10.6	11.3	11.5	7.7	9.5	3.9		1.4	+5.0	+6.9	+9.4	0	+0.8	1.2
		HGB			52.6	49.1	50.9	48.2	29.2	39.0	15.6		5.6	+21.4	+23.6	+34.7	0	+2.1	3.2
		Glucose						←——50 mg/min——→			←——100 mg/min——→			←——150 mg/min——→			←——None——→		

* Abbreviations are as follows: EHBF, estimated hepatic blood flow in ml/min; HV, hepatic venous glucose concentration in mg%; A, arterial glucose concentration in mg %; HV-A, hepatic venous minus arterial glucose concentration in mg %; HGB, hepatic glucose balance in mg/min. Figures without preceding sign indicate net hepatic glucose output. Those preceded by + indicate net hepatic uptake of glucose.

TABLE I—(*Continued*)

Dog	Weight		Control values				Mean control	Time during and after glucose infusion											
			−30	−20	−10	0		10	20	30	40	50	60	70	80	90	100	110	120
no.	*kg*																		
326	17.3	EHBF		207	250	306	254	348	333	312	392	452	521	527	533	491	469	476	500
		HV		126.1	107.7	103.7	112.5	99.7	103.0	111.3	130.0	145.4	146.4	147.3	168.5	165.2	117.9	77.4	55.4
		A		89.0	90.1	85.6	88.2	95.0	102.7	111.3	133.3	151.2	161.8	172.4	177.1	177.4	114.8	74.1	58.4
		HV-A		37.1	17.6	18.1	24.3	4.7	0.3	0	+3.3	+5.8	+15.4	+25.1	+8.6	+12.2	3.1	3.3	+3.0
		HGB		76.8	44.0	55.4	58.7	16.4	1.0	0	+12.9	+26.2	+80.2	+132.3	+45.9	+59.9	14.5	15.7	+15.0
		Glucose						←100 mg/min→			←200 mg/min→			←300 mg/min→			←None→		
327	25.9	EHBF		397	382	418	399	309	357	327	380	312	370	381	381	346	321	323	318
		HV		96.4	98.9	97.6	97.6	99.7	95.8	92.5	99.9	105.5	108.8	113.7	122.9	125.9	104.3	87.7	78.8
		A		83.9	82.1	83.9	83.3	88.7	91.3	93.3	102.7	110.1	115.5	124.9	133.1	140.7	106.3	89.5	79.3
		HV-A		12.5	16.8	13.7	14.3	11.0	4.5	+0.8	+2.8	+4.6	+6.7	+11.2	+10.2	+14.8	+2.0	+1.8	+0.5
		HGB		49.6	64.2	57.3	57.0	42.9	16.0	+2.6	+10.6	+14.4	+24.8	+42.7	+38.9	+51.2	+6.4	+5.8	+1.6
		Glucose						←100 mg/min→			←200 mg/min→			←300 mg/min→			←None→		
522	18.2	EHBF	202	185	189	213	197	210	210	202	212	221	230	240	249	258	259	215	228
		HV	107.1	119.1	101.7	102.5	107.6	116.0	134.9	155.3	191.3	200.4	201.2	195.1	193.3	190.2	185.4	182.1	174.7
		A	83.4	82.6	80.3	79.8	81.5	109.9	142.5	165.5	197.4	204.5	207.6	205.5	210.1	208.6	203.5	198.9	191.0
		HV-A	23.7	36.5	21.4	22.7	26.1	6.1	+7.6	+10.2	+6.1	+4.1	+6.4	+10.4	+16.8	+18.4	+18.1	+16.8	+16.3
		HGB	47.9	67.6	40.5	48.4	51.1	12.8	+16.0	+20.6	+12.9	+9.1	+14.7	+25.0	+41.8	+47.5	+46.9	+36.1	+37.2
		Glucose						←235 mg/min→											
525	20.0	EHBF	208	188	213	222	208	220	255	301	252	273	260	253	204	213	193	159	146
		HV	92.8	89.3	90.5	105.6	94.6	104.9	112.6	120.5	120.0	116.6	111.8	107.9	104.4	104.4	76.6	56.5	52.6
		A	74.6	72.9	72.9	74.2	73.7	100.4	118.8	126.7	125.7	122.8	117.6	115.1	110.4	112.6	71.2	49.1	36.7
		HV-A	18.2	16.4	17.6	31.4	20.9	4.5	+6.2	+6.2	+5.7	+6.2	+5.8	+7.2	+6.0	+8.2	5.4	7.4	15.9
		HGB	37.9	30.8	37.5	69.7	44.0	9.9	+15.8	+18.7	+14.4	+16.9	+15.1	+18.2	+12.2	+17.5	10.4	11.8	23.2
		Glucose						←235 mg/min→									←None→		
610	25.0	EHBF	271	289	262	311	283	269	270	284	370	305	332	224	214	271	260	163	171
		HV	99.7	96.0	96.7	97.7	97.5	105.2	118.3	126.7	133.9	139.9	140.6	132.9	122.5	111.8	85.6	71.7	68.9
		A	83.8	82.3	81.3	82.1	82.4	99.2	119.8	130.0	141.4	146.3	147.1	140.1	127.7	118.8	79.1	61.3	54.6
		HV-A	15.9	13.7	15.4	15.6	15.2	6.0	+1.5	+3.3	+7.5	+6.4	+6.5	+7.2	+5.2	+7.0	6.5	10.4	14.3
		HGB	43.1	39.6	40.3	48.5	42.9	16.1	+4.1	+9.4	+27.8	+19.5	+21.6	+16.1	+11.1	+18.7	16.9	17.0	24.5
		Glucose						←235 mg/min→									←None→		
Mean																			
	20.6	EHBF		219	241	266	248	256	247	266	272	263	285	284	271	271			
		HV		111.2	104.0	101.2	104.4	103.2	107.7	112.1	120.2	128.2	127.0	127.1	129.6	129.2			
		A		84.4	82.2	81.2	82.1	91.5	101.1	106.7	117.2	127.2	128.5	131.4	134.7	135.2			
		HV-A		26.8	21.8	20.0	22.3	11.7	6.6	5.4	3.0	1.0	+1.5	+4.3	+5.1	+6.0			
		HGB		55.7	45.2	49.2	50.7	27.6	12.2	10.6	3.4	1.4	+11.3	+21.6	+17.3	+21.6			
		Glucose						←112 mg/min→			←152 mg/min→			←197 mg/min→					

TABLE II

Changes in hepatic glucose balance from control during a glucose infusion

Dog no.	Mean control	Decrease in hepatic glucose output from control, mg/min*: Minutes after start of glucose infusion 10	20	30	40	50	60	70	80	90	100
35	54.3	22.3	20.7	19.7	28.9	34.6	33.2	38.9	48.4	51.3	
36	68.1	22.8	29.3	11.9	34.3	41.7	55.1	53.3	68.7	68.1	
319	49.2	14.6	14.8	12.3	17.3	27.6	29.3	35.6	36.4	33.6	
513	70.4	33.7	55.2	61.9	64.8	65.4	78.9	69.3	67.4	62.9	65.3
126	10.5	2.2	8.6	17.4	18.3	26.6	28.4	37.3	47.8	44.5	47.6
312	50.9	2.7	21.7	11.9	35.3		45.3	72.3	74.5	85.6	
326	58.7	42.3	57.7	58.7	71.6	84.9	138.9	191.0	104.6	118.6	
327	57.0	14.1	41.0	59.6	67.6	71.4	81.8	99.7	95.9	108.2	
522	51.1	38.3	67.1	71.7	64.0	60.2	65.8	76.1	92.9	98.6	
525	44.0	34.1	59.8	62.7	58.4	60.9	59.1	62.2	56.2	61.5	
610	42.9	26.8	47.0	52.3	70.7	62.4	64.5	59.0	54.0	61.6	
Mean		23.1	38.5	40.1	47.3	49.3	62.0	72.3	68.0	72.3	
p		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

* Value for hepatic glucose balance added to control hepatic glucose output where a net uptake of glucose by the liver was observed.

When the glucose infusion was stopped, hepatic glucose output was usually resumed as the arterial glucose concentration approached or reached control levels (Table I), but in none of the 6 dogs in which hepatic glucose balance was measured during a 30-minute recovery period did hepatic glucose output return to control values. This was particularly striking in 4 animals (Dogs 326, 327, 525, 610) in which hepatic glucose output averaged only 7.8 mg per minute at a time when mean arterial glucose levels had fallen to 57.3 mg per 100 ml, a value 24.6 mg below their control levels. Indeed, in Dogs 326 and 327, net hepatic glucose uptake was still apparent when the arterial glucose level was lower than control.

B. Insulin and glucose infusions. Hepatic glucose output decreased below control values in all 11 dogs during the period of glucose and insulin infusion. Net hepatic uptake of glucose was observed in 6 of the dogs. Failure of glucose infusions to raise the arterial sugar concentration to preinsulin control values probably accounted for the failure of the other 5 dogs to attain a hepatic uptake of glucose. In these dogs insulin infusion lowered mean arterial glucose concentration from a control value of 83.4 to 59.6 mg per 100 ml. When glucose was added to the insulin infusion, the arterial glucose level increased to an average peak value of only 76.5 mg per 100 ml. The detailed data from the 6 animals in which hepatic

TABLE III

Comparison of the arterial glucose concentration, at which net hepatic uptake of glucose was first observed, with control arterial glucose concentration

Glucose infusion			Insulin and glucose infusion			
		Arterial glucose concentration, mg %				
Dog no.	Mean control	Net hepatic uptake first observed	Dog no.	Mean control	Last value with insulin alone	Net hepatic uptake first observed
126	85.0	96.5	1,219	84.9	80.0	126.3
312	60.8	104.7	15	42.5	26.0	92.0
326	88.2	133.3	119	82.5	67.1	69.7
327	83.3	93.3	624	74.8	54.0	95.8
522	81.5	142.5	717	88.5	65.3	91.4
525	73.7	118.8	722	83.4	58.0	82.3
610	82.4	119.8				
Mean	79.3	115.6		76.1		92.9
SD	±8.3	±16.8		±15.6		±17.3

TABLE IV

Effect of insulin and glucose infusions upon hepatic glucose balance *

Dog	Weight		Control values −30	−20	−10	0	Mean control	Last value insulin alone	Time during insulin and glucose infusion 10	20	30	40	50	60	70	80
no.	*kg*															
15	15.4	EHBF		290	332	308	310	236	230	245	267	224	230	257		
		HV		56.0	52.0	52.6	53.5		44.6	57.1	60.3	87.4	114.8	140.2		
		A		43.1	43.4	41.1	42.5	26.0	40.3	54.3	58.3	92.0	118.5	146.8		
		HV-A		12.9	8.6	11.5	11.0		4.3	2.8	2.0	+4.6	+3.7	+6.6		
		HGB		37.4	28.6	35.4	33.8		9.9	6.9	5.3	+10.3	+14.7	+17.0		
		Insulin						←0.026 U/min→								
		Duration insulin alone						←40 min→								
		Glucose							←75 mg/min→			←200 mg/min→				
119	25.5	EHBF		521	512	497	510	606	609	533	583	596	712	664		
		HV		98.0	96.2	94.0	96.1		68.5	72.0	73.7	77.4	82.0	82.0		
		A		85.3	81.1	81.1	82.5	67.1	69.7	74.3	72.8	81.4	85.7	86.5		
		HV-A		12.7	15.1	12.9	13.6		+1.2	+2.3	0.9	+4.0	+3.7	+4.5		
		HGB		66.0	77.3	64.1	69.1		+7.3	+12.3	5.3	+23.8	+26.3	+29.9		
		Insulin						←0.043 U/min→								
		Duration insulin alone						←40 min→								
		Glucose							←100 mg/min→			←200 mg/min→				
1219	18.2	EHBF		166	164	193	174	253	248	275	236	217	277	267		
		HV		100.4	104.7	100.7	101.9		83.6	93.3	100.7	104.4	120.0	130.0		
		A		85.9	84.5	84.2	84.9	80.0	85.6	92.5	99.6	102.1	126.3	138.6		
		HV-A		14.5	20.2	16.5	17.1		+2.0	0.8	1.1	2.3	+6.3	+8.6		
		HGB		24.1	33.1	31.8	29.7		+5.0	2.2	2.6	5.0	+17.4	+23.0		
		Insulin						←0.045 U/min→								
		Duration insulin alone						←40 min→								
		Glucose							←100 mg/min→			←200 mg/min→				
722	27.3	EHBF			302	284	293	344	300	293	201	233	222	223	223	
		HV			100.8	99.4	100.1		80.5	88.7	90.1	93.0	93.3	98.1	94.9	
		A			84.7	82.1	83.4	58.0	82.3	90.3	91.7	95.7	98.4	99.2	99.4	
		HV-A			16.1	17.3	16.7		+1.8	+1.6	+1.6	+2.7	+5.1	+1.1	+4.5	
		HGB			48.6	49.1	48.9		+5.4	+4.7	+3.2	+6.3	+11.3	+2.5	+10.0	
		Insulin						←0.046 U/min→								
		Duration insulin alone						←60 min→								
		Glucose							←235 mg/min→							

* See Table I for abbreviations.

TABLE IV—(*Continued*)

Dog	Weight		Control values −30	−20	−10	0	Mean control	Last value insulin alone	Time during insulin and glucose infusion 10	20	30	40	50	60	70	80
no.	*kg*															
624	15.0	EHBF	231	226	229	256	236	267	321	307	276	279	267	221		
		HV	93.1	90.8	86.1	93.4	90.9		88.6	92.3	99.1	102.9	107.7	108.0		
		A	76.1	75.6	72.9	74.5	74.8	54.0	76.1	95.8	104.0	108.3	115.8	115.0		
		HV-A	17.0	15.2	13.2	18.9	16.1		12.5	+3.5	+4.9	+5.4	+8.1	+7.0		
		HGB	39.3	34.4	30.2	48.4	38.1		40.1	+10.7	+13.5	+15.1	+21.6	+15.5		
		Insulin						←			0.025 U/min			→		
		Duration insulin alone						←60 min→								
		Glucose							←		235 mg/min			→		
717	25.5	EHBF	292	292	266	318	292	288	284	275	308	259	256	221	190	219
		HV	105.7	109.2	104.4	104.1	105.9		89.2	123.2	138.2	146.8	147.6	147.8	152.7	162.6
		A	91.9	92.5	86.6	83.1	88.5	65.3	91.4	123.7	139.7	147.8	149.7	151.6	158.8	164.6
		HV-A	13.8	16.7	17.8	21.0	17.3		+2.2	+0.5	+1.5	+1.0	+2.1	+3.8	+6.1	+2.0
		HGB	40.3	48.8	47.3	66.8	50.8		+6.2	+1.4	+4.6	+2.6	+5.4	+8.4	+11.6	+4.4
		Insulin						←			0.043 U/min					→
		Duration insulin alone						←60 min→								
		Glucose							←		235 mg/min					→
Mean																
	21.2	EHBF		299	301	309	303	332	332	321	312	301	327	309		
		HV		90.9	90.7	90.7	91.4		75.8	87.8	93.7	102.0	110.9	117.7		
		A		76.5	75.5	74.4	76.1	58.4	74.2	88.5	94.4	104.6	115.7	122.3		
		HV-A		14.4	15.2	16.4	15.3		1.6	+0.7	+0.7	+2.6	+4.8	+6.2		
		HGB		42.1	44.2	49.3	45.1		6.5	+3.3	+1.4	+8.9	+16.1	+16.1		
		Insulin						←			0.038 U/min			→		
		Glucose							←	163 mg/min	→	←	218 mg/min	→		

glucose uptake occurred are contained in Table IV and Figure 1. After insulin (0.038 U per minute) had been infused for 40 to 60 minutes, mean control arterial glucose concentration fell from 76.1 to 58.4 mg per 100 ml. As in previous studies reported from this laboratory (1), this decline in arterial glucose concentration was associated with a 52 per cent decrease in mean hepatic glucose output—from 45.1 to 21.6 mg per minute. With the addition of the glucose infusion, a further prompt fall in hepatic glucose output, followed by a net hepatic uptake of glucose, was observed. The magnitude of the hepatic uptake of glucose was similar in the dogs that received only glucose and in those to which both insulin and glucose were administered (Figure 1). The major difference between these two groups in which a positive glucose balance across the liver was observed was *the mean concentration of arterial glucose at which net hepatic glucose uptake first occurred*. When glucose alone was infused, net hepatic uptake was noted at a mean arterial glucose concentration of 115.6 mg per 100 ml (Table III); pretreatment with insulin lowered this value to 92.9. Indeed, in Dog 119, net hepatic uptake of glucose occurred at an arterial glucose level of 69.7 mg per 100 ml, a value 12.8 mg lower than control.

The changes in hepatic glucose metabolism observed in the present experiments were attributed solely to alterations in the arterial-hepatic venous glucose concentration differences, since hepatic blood flow did not change significantly during the course of these studies. The development of a positive arterial-hepatic venous glucose concentration difference, at a time when the arterial glucose concentration was rising, cannot be attributed to sampling errors, since positive glucose balance across the liver also occurred at a time when the arterial glucose concentration had reached a plateau or was falling. If any error in sampling existed, it was in the direction of underestimating the fall in hepatic glucose output. Since in these studies glucose was infused into a vein in the hind limb, the concentration of glucose in inferior vena caval blood would be higher than in blood entering or leaving the liver. Should hepatic venous blood be contaminated by regurgitation from the inferior vena cava, a falsely high value for hepatic venous glucose concentration would result.

2. Disposition of the infused glucose load

The extent to which the liver and the peripheral tissues contribute to the disposition of the glucose load can be approximated from the data. The amount of infused glucose remaining in the glucose pool was estimated by multiplying the change in arterial glucose concentration after glucose infusion by the glucose space. For these calculations it was assumed that the glucose space was 30 per cent of the body weight in the dog (1, 18, 19). That portion of the glucose load which disappeared from the glucose pool, as a result of a change in hepatic glucose metabolism during the glucose infusion, was determined as the amount by which hepatic glucose output decreased from control values. The infused glucose not accounted for either by a decrease in hepatic glucose output or by an increase in the glucose pool was assumed to represent the increase in glucose utilization by the peripheral tissues. Since the filtered load of glucose expected at the blood sugar concentrations obtained in these studies was less than the glucose Tm usually observed in dogs (20, 21), significant renal excretion of glucose seems unlikely. Therefore, no correction was made for possible urinary loss of glucose.

These calculated data are contained in Table V. A mean of 13,810 mg of glucose was added to the glucose pool during a 90-minute period of glucose infusion. Approximately one quarter (3,290 mg) remained in the glucose pool at the end of 90 minutes. Of the 10,520 mg which disappeared from the glucose pool, 48 per cent could be attributed to an altered hepatic glucose output and 52 per cent to an increase in peripheral glucose utilization. The proportion of glucose accounted for by the liver and peripheral tissues varied considerably in different studies. In Dog 35, 80 per cent of the utilized glucose was disposed of by the liver and 20 per cent by peripheral tissues, whereas in Dogs 319 and 610, the liver accounted for only 25 per cent and peripheral tissues for 75 per cent of the glucose utilized. The extent to which the liver and the peripheral tissues utilized glucose was related to the load of glucose infused and, more specifically, to the ratio of glucose infused per minute to control hepatic glucose output. When the ratio of glucose load to initial hepatic glucose output was low, a change in hepatic glu-

TABLE V

Disposition of infused glucose

Dog	Weight	Glucose infused in 90 minutes		− Increase in glucose pool		= Glucose utilized		Disposition of glucose utilized: Decrease in hepatic glucose output		Peripheral* utilization		Fraction of glucose accounted for by	
			Total		Total		Total		Total		Total	Liver	Peripheral tissues
no.	*kg*	*mg/10 kg*	*mg*	*mg/10 kg*	*mg*	*mg/10 kg*	*mg*	*mg/10 kg*	*mg*	*mg/10 kg*	*mg*		
35	16.8	2,680	4,500	785	1,315	1,895	3,185	1,520	2,555	375	630	0.80	0.20
36	25.9	3,470	9,000	1,460	3,790	2,010	5,210	1,355	3,510	660	1,700	0.67	0.33
126	19.1	4,190	8,000	1,405	2,685	2,785	5,315	1,090	2,095	1,685	3,220	0.39	0.61
319	17.2	5,230	9,000	430	745	4,800	8,255	1,190	2,045	3,610	6,210	0.25	0.75
513	19.0	6,840	13,000	1,200	2,275	5,640	10,725	2,780	5,275	2,870	5,450	0.49	0.51
327	25.9	6,950	18,000	1,720	4,460	5,230	13,540	2,260	5,850	2,965	7,690	0.43	0.57
312	12.4	7,250	9,000	3,240	4,010	4,010	4,990	2,800	3,470	1,225	1,520	0.70	0.30
610	25.0	8,560	21,150	1,090	2,730	7,470	18,420	1,870	4,670	5,500	13,750	0.25	0.75
326	17.3	10,400	18,000	2,570	4,625	7,830	13,375	4,670	8,095	3,050	5,280	0.60	0.40
525	20.0	10,575	21,150	1,170	2,335	9,405	18,815	2,475	4,945	6,935	13,870	0.26	0.74
522	18.2	11,600	21,150	3,800	6,940	7,800	14,210	3,210	5,860	4,590	8,350	0.41	0.59
Mean	20.6	6,710	13,810	1,595	3,290	5,115	10,520	2,120	4,360	2,990	6,160	0.48	0.52

* Represents increase in peripheral utilization of glucose above control values.

cose output was of major importance in the disposition of a glucose load. With increasing glucose loads, the peripheral tissues played a proportionately greater role.

DISCUSSION

It is apparent that the liver responds promptly and significantly to a glucose load. Within 10 minutes after beginning an intravenous infusion of glucose in 11 dogs, mean hepatic glucose output fell 46 per cent. The mechanisms responsible for this change are apparently sensitive to very small increments of blood sugar, since this 46 per cent decrease in hepatic glucose output was associated with a rise in mean arterial glucose concentration of only 9.4 mg per 100 ml. As glucose infusion continued, a progressive decrease in hepatic glucose output ensued in all the dogs studied, and in 7 dogs a positive glucose balance across the liver, or net hepatic uptake, was observed.

Theoretically, changes in hepatic glucose balance during the administration of a glucose load may be the consequence of one of the following: first, hyperglycemia per se, in the absence of insulin; second, hyperglycemia in the presence of a fixed amount of insulin; finally, increased secretion of endogenous insulin secondary to arterial hyperglycemia.

The first of these possibilities is best assessed in the insulin-deficient diabetic. In both the diabetic human (22–24) and diabetic dog (25, 26), hepatic glucose output was normal or increased despite the presence of arterial hyperglycemia. A decreased hepatic glucose output or net hepatic uptake of glucose was not observed. By contrast, with comparable arterial glucose concentrations, a marked decrease in hepatic glucose output and significant hepatic uptake of glucose are regularly observed in normal dogs. Furthermore, the response of the liver to a glucose load was impaired in dogs made diabetic by partial pancreatectomy and administration of alloxan (26). Thus, the arterial glucose concentration at which the liver stopped putting out glucose and started taking it up was elevated; the more severe the diabetes, the higher the blood glucose level required for hepatic glucose uptake; finally, elevation of blood glucose to as high as 500 mg per 100 ml did not result in hepatic uptake of glucose in some dogs. These observations indicate that hyperglycemia per se, in the absence of insulin, cannot explain the alterations in hepatic glucose balance observed in the present study.

The possibility that hyperglycemia in the presence of a fixed amount of insulin is responsible for the changes in hepatic glucose balance was not assessed in the present studies, since endogenous insulin secretion probably increased during glucose loading (3, 4). Nevertheless, this possibility was suggested by the studies of Soskin, Allweiss and Cohn (27), based on glucose tolerance tests in dogs. In the studies of Soskin and

colleagues, a constant blood sugar was maintained in depancreatized dogs by the constant infusion of both insulin and glucose for at least 1 to 2 hours. When these infusions were continued, the subsequent injection of an additional glucose load resulted in "normal" glucose tolerance tests in most of these animals. Since further secretion of endogenous insulin was impossible, an increase in insulin secretion was not considered necessary for the normal disposition of a glucose load. When the pancreas was left intact but the liver removed, glucose loading did not result in normal glucose tolerance, findings which supported their conclusions. Although Soskin's experiments reveal that normal glucose utilization is dependent upon insulin, they do not prove that it is dependent upon the presence of a fixed amount of insulin. Despite the administration of insulin by constant infusion, it is possible that the rate of infusion exceeded the rate of degradation. Therefore, insulin may have been accumulating. Since plasma insulin concentrations were not measured, progressively increasing insulin levels were not excluded. Furthermore, failure to attain normal glucose tolerance in hepatectomized animals with an intact pancreas does not exclude increased insulin secretion as an important factor in obtaining normal glucose tolerance. Soskin's own observations, and those of the present study, indicate that the liver accounts for a significant portion of a glucose load. It seems reasonable that removal of this quantitatively important insulin-responsive organ, even in the presence of increased amounts of insulin, accounted for the impaired glucose tolerance observed by Soskin and co-workers in their hepatectomized dogs. As Park and associates have shown (28), the rate of glucose transport from extracellular fluid into muscle cells is dependent upon extracellular glucose concentration. In the presence of insulin, the rate of glucose transport is increased for any given glucose concentration. It is likely that a significant number of glucose transport sites had been affected by insulin during the prolonged insulin infusion in Soskin and co-workers' studies (*27*). Under this circumstance, normal glucose disappearance after glucose loading would be expected.

There appears to be considerable evidence to support the view that increased secretion of endogenous insulin, secondary to arterial hyperglycemia, accounts for a decrease in hepatic glucose output and subsequent net hepatic uptake of glucose. It has been demonstrated that insulin is released promptly into pancreatic venous blood after administration of glucose (3, 4). The slow intravenous infusion of insulin produces a prompt fall in hepatic glucose output, even within 10 minutes (1). In the present studies insulin administration prior to glucose loading resulted in a lowering of the arterial glucose concentration at which net hepatic uptake of glucose was observed. The impaired response of the liver to glucose infusion in diabetic dogs is rapidly improved toward normal by insulin administration (*26*). In the presence of increased insulin secretion, the level of blood sugar probably also determines the extent to which the liver and peripheral tissues dispose of a glucose load. Nevertheless, significant insulin effects on the liver, independent of blood glucose levels, were observed in the present studies. Thus, in Dogs 522, 525 and 610, net hepatic uptake of glucose was as great or greater at a time when the blood glucose concentration was falling, and the arterial glucose level was lower than it was earlier in the study when arterial blood sugar was rising. Indeed, in Dog 119, pretreated with insulin, a positive glucose balance across the liver was observed at arterial glucose concentrations even lower than control. Finally, the persistence of a decreased hepatic glucose output, despite a fall in arterial glucose concentration to values lower than control during the recovery period in four of the dogs in these studies (*326*, *327*, 525, 610), suggests a continuing hepatic action of endogenous insulin, previously secreted during the period of hyperglycemia.

A number of previous investigations has been concerned with the effects of glucose loading on hepatic glucose metabolism. The changes in hepatic glucose balance observed during glucose infusion in our studies in the dog closely resemble the findings of Soskin, Essex, Herrick and Mann (5). Their observations, like our own, are based upon direct measurements of hepatic blood flow and the concentration differences of glucose in blood entering and leaving the liver. Most of their data were obtained in acutely operated dogs given a single rapid intravenous injection of glucose. In only two of their studies was glucose administered by constant intravenous infusion.

Studies in which glucose concentration in arterial, portal and hepatic venous blood was determined, without concomitant measurements of hepatic blood flow, also provide evidence for, but no quantitation of, a decrease in hepatic glucose output and net hepatic glucose uptake during glucose feeding (6) and infusion (7). Bondy, James and Farrar (29), and Myers (23), utilizing hepatic vein catheterization in man, found evidence of glucose retention in the splanchnic viscera after the intravenous administration of glucose. In such studies, however, as Bondy has pointed out, it is impossible to differentiate changes in hepatic glucose metabolism from over-all splanchnic metabolism.

Whereas the above results are in keeping with the findings of the present experiments, studies based on two different isotope dilution techniques are either inconclusive or fail to detect a hepatic response. In the single injection method used by both Searle and Chaikoff (8) and Reichard, Friedmann, Maass and Weinhouse (9), changes in the specific activity of plasma glucose were followed after administration of a tracer dose of glucose-C^{14}. The exponential decline in specific activity during the control period was superseded by a "plateau" after the administration of an unlabeled glucose load. This change in plasma glucose specific activity was attributed to a cessation of hepatic glucose output (8, 9). Berson, Weisenfeld and Pascullo conducted similar experiments in rabbits (10) and have challenged the validity of this interpretation. On theoretical grounds, these authors concluded that "a short period in which the specific activity in the blood remains constant following unlabeled glucose injection is an obligatory consequence of the kinetics of glucose mixing and distribution even if there is no inhibition of glucose production." The studies of Steele, Bishop and Levine (11) also show that the kinetics of mixing of injected glucose may result in a plateauing of glucose specific activity under circumstances in which a decrease in hepatic glucose output could not have occurred. In their studies in eviscerated animals, hepatic glucose output was replaced by a constant infusion of unlabeled glucose. Plateauing of the specific activity of plasma glucose was observed in these animals after administration of a glucose load, even though the constant infusion of glucose was maintained.

To overcome these objections to the single injection technique, the priming dose-constant infusion method of administering glucose-C^{14} has been utilized by Steele and Marks in two experiments (12) and by de Bodo and associates in two experiments (13). No evidence of a decrease in hepatic glucose output after glucose loading was noted in their studies. As was pointed out in a previous publication (1), a major drawback to the isotope dilution studies is that they can measure only new glucose production by the liver. Such techniques cannot measure hepatic glucose utilization or differentiate it from peripheral glucose utilization. The failure of the priming dose-constant infusion studies to detect an alteration in hepatic glucose output after glucose loads, therefore, cannot be considered as unequivocal evidence that such an effect does not occur.

The data accumulated in this and previous studies (1) indicate the presence of a sensitive, integrated system geared to the metabolism of ingested carbohydrate by the liver. The organization of this system appears to be well suited structurally by virtue of the anatomical relationships of the gastrointestinal tract, pancreas, and liver to the portal vein. Thus, glucose absorbed from the intestine passes into portal venous blood where it mixes with insulin released from the pancreas. Glucose and insulin, in concentrations higher than those possible for any other organ, are then carried directly to the liver, a situation which appears to be ideal for the hepatic metabolism of glucose. In the present studies, conducted in dogs with end-to-side portacaval shunts, not only did the insulin secreted in response to the glucose load bypass the liver initially, but the same was true of the glucose that was infused into a peripheral vein. Under these circumstances, the liver was exposed to relatively lower concentrations of both insulin and glucose than would have occurred after the oral ingestion of a carbohydrate load in intact animals. Nevertheless, the total quantity of glucose accounted for by an alteration in hepatic glucose metabolism was appreciable. Indeed, of the glucose which disappeared from the glucose pool, almost as much was accounted for by a change in hepatic glucose metabolism as by an increase in peripheral glucose utilization. Normally, when the liver alone is exposed to the

high concentrations of glucose and insulin present in portal blood after feeding, it would seem reasonable to expect the liver to account for an even greater proportion of a glucose load.

These remarks should not be construed to minimize the importance of the peripheral tissues in the disposition of a carbohydrate load. Previous studies from this laboratory have indicated that the proportion of the secreted insulin which is bound to the liver during the initial transhepatic circulation continually decreases when the intact subject receives carbohydrates (30, 31). The peripheral tissues are thereby supplied with a progressively increasing proportion of the secreted insulin. Indeed, in the present studies with prolongation of the glucose infusion and with larger carbohydrate loads, the peripheral tissues played a proportionately greater role in the disposition of this infused carbohydrate. It should be emphasized that *both* the liver and the peripheral tissues play important roles in carbohydrate utilization. Furthermore, these roles are directly affected by endogenous insulin, which under physiologic conditions is secreted in response to hyperglycemia.

SUMMARY AND CONCLUSIONS

The effect of glucose infusions upon hepatic glucose balance was appraised in dogs with portacaval shunts. This preparation was chosen since it permits determination of changes in hepatic rather than in splanchnic glucose metabolism.

The data indicate that the liver responds to a glucose load first by a decrease in net glucose output, and then by an actual uptake or storage of glucose. These changes occur quickly and are apparently mediated by endogenous insulin which is secreted in response to a rising blood sugar. Quantitatively, these hepatic effects are significant, the liver accounting for almost as much of the infused glucose load as peripheral tissues.

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THE METABOLISM OF CITRATE-C^{14} IN NORMAL AND IN FLUOROINHIBITOR-POISONED RATS *

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A cyclical exchange of citrate has been shown to exist in the intact organism, with the blood plasma serving as the transporting medium. *In vivo* studies have indicated a considerable uptake of citrate by the liver (1) and kidney (2), and a release of citrate into the blood by the peripheral tissues (1, 3) and intestine (4, 5). Interest in the blood citrate flux has centered primarily around the importance of this intermediate in carbohydrate metabolism, in its relationship to calcium mobilization and deposition in bone (6), and in its role as a chelating agent for calcium in the urine (7, 8).

The studies reported here have been concerned with an attempt to elucidate the metabolic fate of the blood citrate in rats with the aid of radioactive citrate. The data, obtained by following the pattern of respiratory $C^{14}O_2$ and the urinary excretion of radioactive carbon after the intravenous administration of sodium citrate-1,5-C^{14}, indicated a rapid turnover of the blood citrate. Three hours after injection of the labeled citrate, approximately 90 per cent of the radioactivity was recovered in the respiratory CO_2 and in the urinary citrate. Evidence for the utilization of blood citrate as a precursor of tissue components was provided by assaying the C^{14} content of protein and lipid of kidneys and liver after intravenous citrate-C^{14} administration.

In an effort to define further the metabolic fate of the blood citrate, inhibitors of citrate oxidation were administered prior to citrate-C^{14} administration. Peters (9) has shown that fluoroacetate inhibits the enzyme, aconitase, by virtue of its conversion to fluorocitrate. As a consequence of aconitase inhibition, the metabolism of citrate to isocitrate in the Krebs tricarboxylic acid cycle is blocked. In our experiments both fluoroacetate and fluorocitrate influenced the fate of the administered citrate-C^{14}. In the intact animal the effects of each of the inhibitors were strikingly different: fluroacetate resulted in a decreased, and fluorocitrate resulted in an increased urinary excretion of citrate-C^{14}. Tissue slice experiments confirmed this difference in the action of the inhibitors. In contrast to the postulate of Peters, the *in vitro* data are consistent with the view that fluoroacetate is also an inhibitor of the condensing enzyme.

MATERIALS AND METHODS

Fed, female Sprague-Dawley rats, weighing between 185 and 235 g, were used. Citric acid-1,5-C^{14} was obtained from Tracerlab, Inc., and sodium acetate-1-C^{14} from Volk Radiochemical Co. Sodium fluoroacetate [1] was purchased from Bios Laboratories, Inc. Barium fluorocitrate, synthesized by the method of Rivett (10), was obtained from the Sonbert Chemical Co., Brooklyn, N. Y.; this preparation contained less than 1 per cent barium citrate by specific analysis. Before use, the barium salt was dissolved in dilute HCl, the barium was precipitated with saturated sodium sulfate and the supernatant fluid was neutralized with dilute NaOH. Solutions of the fluoroinhibitors were freshly prepared prior to use.

In the *in vivo* experiments, sodium citrate-1,5-C^{14} in isotonic saline was given intravenously in a volume of 0.3 ml. Collection of respiratory CO_2 was accomplished by drawing CO_2-free air through a glass metabolism jar containing the rat, and the respiratory CO_2 was trapped in NaOH. Barium carbonate precipitates were prepared from aliquots of the NaOH solutions. After the $BaCO_3$ was washed with water and then alcohol, it was plated and counted in a Nuclear-Chicago gas-flow counter with a Micromil window. Urine collections were made up to standard volume, and aliquots were taken for direct plating and for conversion of the carboxyl groups of the urinary citrate to CO_2 with ceric sulfate (11); the CO_2

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[1] Elementary analysis: $C_2H_2O_2F$ Na (100.0); calculated, C 24.0, H 2.0, F 19.0; found, C 24.3, H 2.1, F 18.2; performed by the Schwarzkopf Microanalytical Lab., Woodside, N. Y.

was collected in NaOH and subsequently converted to $BaCO_3$ for radioactive assay as stated above. Because of the lack of absolute specificity of the ceric sulfate method for measurement of citrate in the presence of other intermediates, another method was used to verify the specific activity of the urinary citrate. Interfering inorganic salts of urine were removed by passage through a Dowex-2 column (12). The column eluate was taken to dryness, made up to volume with water and an aliquot subjected to ascending chromatography on Whatman no. 4 paper in a *n*-butanol : acetic acid : water system (120 : 30 : 50). The citric acid was located by means of an indicator spray (13), the spot was eluted and aliquots were taken for determination of citrate and for conversion of the carboxyl groups to $BaCO_3$ as described above. The results of this technique for measuring the specific activity of the citrate were in good agreement with those obtained by direct treatment of urine with ceric sulfate.

Three hours after administration of the radioactive citrate, the rats were sacrificed by cervical fracture, and the kidneys and liver were removed and weighed. Portions of each of the organs were taken for isolation of lipid (14) and protein (15). The lipid was plated on planchets containing a circle of lens paper, and the protein was counted as a powder. All radioactive samples were corrected for self-absorption and the values converted to infinite thickness.

In the *in vivo* fluoroinhibitor experiments, fluoroacetate or fluorocitrate was injected intraperitoneally, in a volume not exceeding 0.4 ml, 30 minutes prior to citrate-C^{14} administration. The respiratory CO_2 and urine were collected, plated and counted as described above. After the rats were sacrificed, weighed portions of kidney, liver, heart, and spleen were homogenized in ice-cold 5 per cent trichloroacetic acid. After centrifugation the citrate concentration of the supernatant fluid was estimated by the Eliott modification of the Natelson, Pincus and Lugovoy method (16).

In vitro studies of liver and kidney slices, prepared with a Stadie-Riggs microtome, were carried out in 25-ml Erlenmeyer flasks with a sealed-in center well containing filter paper saturated with 0.2 ml of 5 per cent NaOH. Slices of liver (approximately 400 mg per vessel) and kidney (approximately 150 mg per vessel) were incubated in 3 ml of Krebs-Ringer phosphate buffer containing 200 mg per 100 ml glucose, pH 7.4. After a 15-minute equilibration period at 37°C in a Dubnoff metabolic shaker under 100 per cent O_2, a trace dose of citrate-1,5-C^{14} or acetate-1-C^{14} was added to the flasks. The closed flasks were then incubated for 60 minutes and the $C^{14}O_2$ produced was trapped in the NaOH of the center well. Fluoroacetate was present in a concentration of 2.5×10^{-4} M and fluorocitrate in a concentration of 5×10^{-4} M. At the end of the incubation period, the center well NaOH was collected, carrier $NaHCO_3$ was added, and the samples were converted to $BaCO_3$ for plating and counting. The tissue was homogenized in the incubating medium and flask washings, and the protein was precipitated with an equal volume of 10 per cent trichloroacetic acid.

Fractionation of liver and kidney tissues to determine the intracellular distribution of citrate was carried out by a modification of the method of Schneider (17). A 10 per cent (wt/vol) homogenate of the tissue was prepared in 0.25 M sucrose with a motor-driven Teflon pestle or with a Dounce homogenizer at 0°C. A portion of the whole homogenate was stored at 0° until the end of the separation procedure. Further fractionation of the whole homogenate was accomplished by centrifuging at 700 G for 10 minutes at 5°C. The supernatant solution was removed and the sediment was resuspended in 0.25 M sucrose, homogenized, and centrifuged at 700 G for 10 minutes. This supernatant was combined with the supernatant from the first centrifugation and the sediment was resuspended in water and homogenized; this nuclear fraction was stored at 0° until the end of the fractionation procedure. The combined supernatants were then spun at 5° for 10 minutes at 5,000 G to sediment the mitochondria. The supernatant and the fluffy layer containing microsomes and soluble cytoplasm were removed, and this fraction is referred to as the *extramitochondrial fraction*. The sediment, representing the mitochondrial fraction, was resuspended and homogenized in distilled water. Appropriate aliquots of each of the fractions were added to equal volumes of 10 per cent trichloroacetic acid and the protein was removed by centrifugation. Citrate determinations in the presence of sucrose were carried out by a modification of the pentabromacetone procedure (18).

RESULTS

The fate of intravenously administered citrate-C^{14} in the intact rat. Initially, the rates of conversion of 4 μg of citrate-C^{14} (1.1×10^5 cpm) and 125 μg of citrate-C^{14} (5.6×10^6 cpm) to respiratory CO_2 and urinary citrate were compared in order to determine whether the larger quantity of citrate could be administered without significantly altering the physiological state (Table I). The rate of conversion of the radioactive carbon of citrate-C^{14} to respiratory CO_2 and the percentage of the administered label in the urinary citrate were independent of the amount of citrate-C^{14} ad-

TABLE I

The percentage of counts after 3 hours in the respiratory CO_2 and urinary citrate as a function of the quantity of citrate-C^{14} injected *

Citrate injected	Respiratory CO_2	Urinary citrate
μg		
4	54.4 ± 4.9 [5]	26.8 ± 3.0 [6]
125	55.5 ± 1.0 [5]	34.4 ± 2.4 [5]

* The number of rats in each series is indicated in brackets; the values are the mean ± standard error.

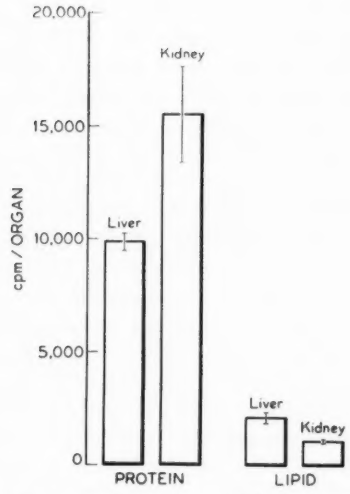

FIG. 1. THE INCORPORATION OF C^{14} INTO TOTAL LIPID AND PROTEIN OF THE RAT KIDNEY AND LIVER 3 HOURS AFTER ADMINISTRATION OF CITRATE-C^{14}. The values are the means of 4 rats ± standard error.

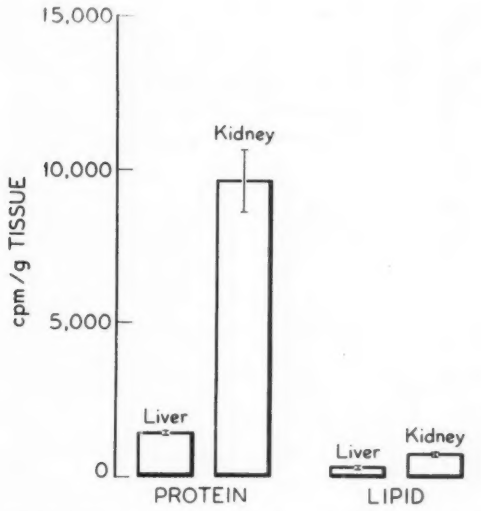

FIG. 2. THE INCORPORATION OF C^{14} INTO LIPID AND PROTEIN OF RAT LIVER AND KIDNEY 3 HOURS AFTER ADMINISTRATION OF CITRATE-C^{14}, EXPRESSED AS COUNTS PER MINUTE PER GRAM TISSUE. The values are the means of 4 rats ± standard error.

ministered (a t test of differences for each of the parameters revealed $p > 0.05$). Eighty to 90 per cent of the administered radioactivity was recovered in the 3-hour period. Since these results indicated that 125 μg of citrate could be administered intravenously without gross distortion of the metabolic fate of the blood citrate, this quantity was used in all subsequent *in vivo* experiments.

After 3 hours, approximately 1 per cent of the radioactive carbon of citrate-C^{14} was incorporated into the protein and lipid moieties of kidney and liver. The incorporation of the C^{14} into the protein of these organs was far greater than that incorporated into the lipid (Figure 1). The C^{14} content of kidney protein was not significantly different from that of liver protein ($p > 0.05$), but the C^{14} content of kidney lipid was less than that of liver lipid ($p < 0.025$). The data also indicate (Figure 2) that incorporation of the radioactive carbon of citrate-C^{14} into kidney tissue components was far greater than that incorporated into liver tissue components when expressed on a gram-weight basis ($p < 0.001$ for lipid, $p < 0.001$ for protein).

Effect of fluoroacetate and fluorocitrate in the intact rat. In the intact animal, fluorocitrate differed from fluoroacetate with respect to its effect on the metabolism of citrate. The effectiveness of these substances as inhibitors of metabolism *in vivo* is reflected in the accumulation of tissue citrate (Table II); 3.5 hours after the intraperitoneal administration of fluoroacetate at a level

TABLE II

Tissue citrate *

Fluoroinhibitor	None [4]	Fluoroacetate [4] (2.5 mg/kg)	Fluorocitrate [3] (20 mg/kg)	Fluorocitrate [3] (40 mg/kg)
Heart	15.6 ± 6.2	504 ± 83	43.2 ± 4.2	50.8 ± 12
Kidney	32.2 ± 8.1	1,118 ± 65	88.4 ± 6.1	175 ± 24
Spleen	33.2 ± 5.6	1,011 ± 46	84.1 ± 15.8	193 ± 11
Liver	23.1 ± 6.5	336 ± 50	64.8 ± 10.5	110 ± 8

* Accumulation of citrate (in μg/g wet weight tissue) in female rat tissues 3.5 hours after the intraperitoneal administration of fluoroinhibitor. The values are the mean ± standard error. The number of animals in each group is indicated in brackets.

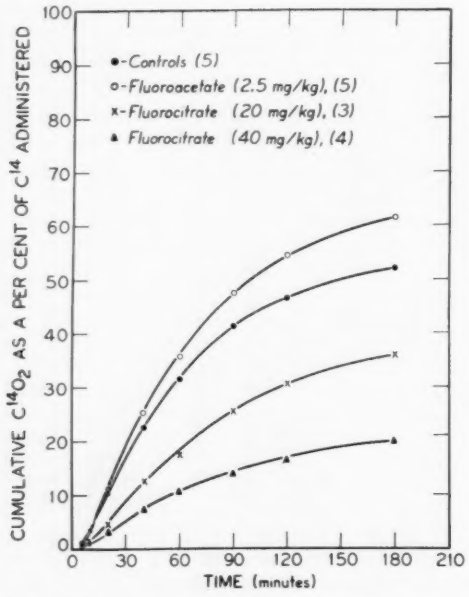

FIG. 3. THE CONVERSION OF INTRAVENOUSLY ADMINISTERED CITRATE-1,5-C^{14} TO $C^{14}O_2$ IN NORMAL RATS AND IN RATS PRETREATED WITH FLUOROINHIBITORS. The fluoroinhibitors were injected intraperitoneally 30 minutes prior to citrate-C^{14} administration. The values are the means for the number of animals (indicated in parentheses).

of 2.5 mg per kg (25 μmoles per kg) or fluorocitrate at a level of 20 and 40 mg per kg (50 and 100 μmoles per kg), the concentration of citrate in the tissues was increased over the control, in agreement with the data of Gal, Peters and Wakelin (19). There was approximately a 30-fold increase in heart, kidney and spleen citrate and a 15-fold increase in liver citrate in the fluoroacetate-treated animals; fluorocitrate increased the tissue citrate concentration three- to sixfold. Despite the markedly greater inhibition of citrate metabolism by fluoroacetate when tissue citrate concentrations were used as a guide, the conversion of intravenously administered C^{14}-labeled citrate to respiratory $C^{14}O_2$ was greater ($p < 0.01$) in the fluoroacetate-treated than in the control animals (Figure 3). On the other hand, fluorocitrate administration resulted in a depression ($p < 0.001$ at 20 and 40 mg per kg) of respiratory $C^{14}O_2$ from labeled citrate.

The effect of these fluoroinhibitors on endogenous citrate metabolism and the fate of administered citrate-C^{14} was further studied by examining the urine and blood of these animals (Table III). After 3 hours, approximately 35 per cent of the administered C^{14} was found in the urine, whereas 50 per cent of the radioactivity was found in the urine of fluorocitrate-poisoned animals and 15 per cent in the urine of the fluoroacetate-poisoned animals. Essentially all of the radioactivity of the urine after citrate-C^{14} administration was present in the carboxyl groups of the urinary citrate. Fluoroacetate treatment did not affect the quantity of citrate excreted in the urine, but fluorocitrate administration resulted in a four- to fivefold increase in urinary citrate excretion. Blood citrate levels were increased two to three times over normal when the animals received either fluoroacetate or fluorocitrate. These findings indicated that the difference in the pattern of respiratory $C^{14}O_2$ after citrate-C^{14} administration in the fluoroinhibitor-treated rats was the result of alterations in the renal clearance of the labeled citrate as well as inhibition of citrate oxidation.

In vitro activity of fluoroinhibitors on citrate and acetate metabolism. In view of the discrepancy between fluoroacetate and fluorocitrate action on citrate metabolism in the intact animal, *in vitro* studies were carried out with slices of rat liver and kidney (Table IV). It has been suggested by Gal and co-workers (19) that only two of the four isomers of chemically synthesized fluorocitrate are biologically active; thus the effective molar concentrations of the two fluoroinhibitor compounds studied *in vitro* were the same, 2.5×10^{-4} M. At this concentration there was an accumulation of citrate in the incubation vessels when either of the fluoroinhibitors was

TABLE III

The blood and urine citrate concentration in normal and fluoroinhibitor-treated rats *

	Normal	Fluoroacetate (2.5 mg/kg)	Fluorocitrate (20 mg/kg)
Blood citrate (μg/ml)	33.2 ± 3.4	91.6 ± 6.3	66.0 ± 3.6
Urine citrate (mg/3 hrs)	8.1 ± 0.7	6.1 ± 1.3	35.4 ± 1.4
% Counts injected			
Urine	37.6 ± 2.0	15.4 ± 2.3	49.6 ± 3.6
Urinary citrate	34.4 ± 2.4	12.7 ± 1.9	53.0 ± 1.4

* Citrate-1,5-C^{14} was injected intravenously 30 minutes after the intraperitoneal administration of the fluoroinhibitor, and the urine was then collected for 3 hours. Five rats were used in each series and the values represent the mean ± standard error.

TABLE IV

The effect of fluoroacetate and fluorocitrate on in vitro metabolism of liver and kidney *

	Citrate-1,5-C^{14}		Acetate-1-C^{14}	
	Citrate produced (μg/g wet wt)	$C^{14}O_2$ produced (cpm/mg wet wt)	Citrate produced (μg/g wet wt)	$C^{14}O_2$ produced (cpm/mg wet wt)
Liver				
Control	26 ± 2	75 ± 4	2 ± 3	139 ± 8
Fluoroacetate	122 ± 11	53 ± 2	93 ± 15	66 ± 4
Fluorocitrate	438 ± 12	7 ± 0.5	423 ± 28	18 ± 0.7
Kidney				
Control	16 ± 10	459 ± 25	13 ± 12	796 ± 25
Fluoroacetate	567 ± 30	393 ± 30	532 ± 24	569 ± 20
Fluorocitrate	938 ± 50	78 ± 28	867 ± 45	638 ± 25

* The values represent the mean ± standard error of 8 observations.

present; fluorocitrate was about twice as effective as fluoroacetate in this regard for kidney slices and about four times as effective with liver slices. It should be noted that the conversion of citrate-C^{14} and acetate-C^{14} to $C^{14}O_2$ by kidney slices was approximately six times greater than with liver slices in the absence of inhibitors. Similarly, the uptake of unlabeled citrate, present in the incubation medium at a concentration of 5 mmoles per L by kidney slices was about six to seven times that found with liver slices (20). Differences in tissue responsiveness to these inhibitors are pointed up by the fact that fluorocitrate had a more striking effect on liver tissue than did fluoracetate in regard to the conversion of either citrate-C^{14} or acetate-C^{14} to $C^{14}O_2$. In contrast, the conversion of acetate-C^{14} to $C^{14}O_2$ by kidney tissue was inhibited to the same extent when either fluoroacetate or fluorocitrate was present. Citrate-C^{14} conversion to $C^{14}O_2$ by kidney was inhibited to a much greater extent by fluorocitrate than by fluoroacetate. With liver slices, fluoroacetate proved to be a more effective inhibitor of acetate metabolism than of citrate metabolism.

TABLE V

Distribution of citrate in the subcellular fractions of kidney and liver prepared from rats pretreated with fluoroinhibitors *

	Fluoroacetate		Fluorocitrate	
	μg cit./g tissue	% tissue cit.	μg cit./g tissue	% tissue cit.
Liver				
Whole homogenate	326	100	526	100
Nuclear fraction	48.0	14.7	53.7	10.2
Mitochondrial fraction	202	62.0	338	64.3
Extramitochondrial fraction	74	22.7	196	22.7
Kidney				
Whole homogenate	630	100	597	100
Nuclear fraction	57	9.1	49.7	8.3
Mitochondrial fraction	180	28.6	134	22.5
Extramitochondrial fraction	320	50.8	365	61.1

* The fluoroacetate (2.5 mg/kg) and fluorocitrate (20 mg/kg) were administered intraperitoneally and the animals were sacrificed 2 hours later.

Intracellular distribution of citrate after fluoroinhibitor administration. Additional information concerning citrate metabolism was obtained by studying citrate accumulation in subcellular components of kidney and liver tissue after administration of each of the fluoroinhibitors to rats (Table V). In control animals, the preponderance of tissue citrate was localized in the mitochondrial fraction of kidney and liver. After administration of fluoroacetate to rats, approximately 60 per cent of the liver citrate was found in the mitochondrial fraction, in confirmation of the data of Schneider, Striebich and Hogeboom (18). In most instances the remainder of the tissue citrate could be accounted for in the nuclear fraction and in the fraction not sedimented by centrifuging at 5,000 G for 10 minutes. Essentially the same distribution of citrate in liver tissue was found in the fluorocitrate-treated animal. In contrast, about one-half to two-thirds of kidney citrate was recovered in the fraction not sedimenting at 5,000 G for 10 minutes when either fluoroacetate or fluorocitrate was administered to the animal; the balance of the tissue citrate was found in the mitochondrial and nuclear fractions. Tissue fractionation with 0.88 M sucrose, isotonic saline, 7.3

per cent polyvinylpyrrolidone-0.25 M sucrose (21) resulted in essentially the same distribution of tissue citrate after fluoroinhibitor administration as did 0.25 M sucrose.

DISCUSSION

These investigations revealed that the blood citrate is metabolized at a rapid rate, as indicated by following the fate of intravenously injected radioactive citrate. By 3 hours, 55 per cent of the label was found in the respiratory CO_2; of the total radioactivity administered, 35 per cent was recovered in the urinary citrate. Thus, about 85 per cent of the citrate-C^{14} that remained in the animal was metabolized to $C^{14}O_2$ in 3 hours. This rate approaches that seen after administration of acetate-1-C^{14} to rats (22). The rapid turnover of the blood citrate dictates that there is an equally rapid outflux from the tissues. A similar respiratory $C^{14}O_2$ pattern was obtained by Lussier (23) after the intraperitoneal injection of 2 mg radioactive citric acid; only 10 per cent of the administered label was recovered in the urine in 8 hours, in contrast to the findings reported here. Since urinary citrate-C^{14} excretion is markedly depressed when 1.5 mEq of HCl is introduced into the stomach prior to injection of citrate-C^{14} (20), it seems likely that the low levels obtained in the earlier experiments were due to the administration of the radioactive compound as the free acid.

Evidence for the utilization of the blood citrate as a precursor of tissue components is provided by the finding that approximately 1 per cent of administered C^{14} as citrate-C^{14} was incorporated into lipid and protein of the kidneys and liver (Figure 1). Kidney was much more active in this respect than liver when the data are calculated as counts per minute incorporated per gram of tissue (Figure 2). It is likely that an appreciable portion of the injected radioactivity that remains unaccounted for was taken up by bone and later released or metabolized (23).

Peters (9) has demonstrated that fluoroacetate is converted to fluorocitrate by biological systems, with consequent inhibition of the enzyme, aconitase. Other studies (19), however, indicated that these two compounds did not result in equivalent inhibitory activities when administered to rats. It was shown that fluorocitrate, but not fluoroacetate, resulted in an appreciable accumulation of citrate in the livers of male rats, in contrast to the findings in female rats (Table II). Further, citrate accumulation in the heart was considerably greater after fluoroacetate than fluorocitrate administration. The present study of the effect of fluoroacetate and fluorocitrate on citrate metabolism provides additional evidence for the divergent action of these two inhibitors *in vivo* and in tissue slices.

In the intact animal, conversion of citrate-C^{14} to respiratory $C^{14}O_2$ was enhanced by pretreatment with fluoroacetate despite inhibition of citrate metabolism, as evidenced by accumulation of citrate in the tissues. In animals pretreated with fluorocitrate, conversion of citrate-C^{14} to respiratory $C^{14}O_2$ was depressed; in this case the amount of citrate that had accumulated within the tissues was far less than that observed in the fluoroacetate-treated rats. These seemingly paradoxical findings were partially resolved upon analysis of the urinary excretion of the labeled citrate. In the one instance, fluoroacetate markedly inhibited the urinary excretion of the radioactive carbon, while fluorocitrate treatment resulted in an increased urinary excretion of radioactive carbon. Because of this, more radioactive citrate was available for oxidation to respiratory $C^{14}O_2$ in the fluoroacetate-treated rat than in the normal and fluorocitrate-treated rat. In the fluoroacetate-poisoned rat one might expect that the administered radioactive citrate would be diluted to a large extent by the increased concentration of citrate in the tissues, with a consequent decreased rate of oxidation of the citrate-C^{14} to $C^{14}O_2$. Since this was not the case, the existence of more than one pool of citrate within the organism is suggested.

Another difference in the action of these fluoroinhibitors was noted; fluorocitrate, unlike fluoroacetate, caused striking increases in total urinary citrate excretion. Since the blood citrate levels were markedly elevated in both the fluoroacetate- and fluorocitrate-treated rats, the data indicate that the renal tubular mechanism for handling citrate is affected diversely by each of the fluoroinhibitors that was tested.

Further confirmation of the difference in tissue responsiveness to fluorocitrate and fluoroacetate was obtained from *in vitro* tissue-slice ex-

periments. The data indicate that fluoroacetate served as a more effective inhibitor of acetate oxidation than of citrate oxidation in both liver and kidney slices. This observation is in agreement with the finding of decreased acetate utilization by slices of heart, kidney and liver when fluoroacetate was present in the incubation medium (24). In other experiments Kalnitsky (25) demonstrated that the presence of fluoroacetate at a concentration of 0.001 M completely inhibited acetate utilization and only slightly inhibited citrate utilization by rabbit kidney cortex homogenates. A possible explanation of these findings is provided by the fact that fluoroacetyl coenzyme A behaves as a strong competitive inhibitor of the condensing enzyme (26).

Schneider and co-workers (18) demonstrated that the accumulation of liver citrate after fluoroacetate administration to the animal could be found primarily in the mitochondrial fraction. Other experiments have shown that aconitase, the enzyme competitively inhibited by fluorocitrate, exists in two forms, each displaying characteristic pH optima (27, 28). These observations prompted an investigation of the effect of fluoroacetate and fluorocitrate on the intracellular distribution of citrate in liver and kidney tissue. The present studies confirmed the localization of citrate in liver mitochondria after fluoroacetate poisoning and, in addition, showed that fluorocitrate resulted in essentially the same intracellular distribution of citrate. Analysis of kidney tissue of animals treated with fluoroacetate or fluorocitrate revealed a different distribution of citrate in the subcellular fractions from that of liver. Pretreatment with fluoroacetate or fluorocitrate resulted in essentially the same distribution of citrate within the subcellular fractions of kidney tissue. No evidence is available to provide an explanation for the difference between liver and kidney tissue in the subcellular localization of citrate after fluoroinhibitor treatment.

SUMMARY

The turnover of the blood citrate is extremely rapid as measured by the conversion of intravenously administered citrate-C^{14} to respiratory $C^{14}O_2$. Approximately 85 per cent of the radioactive carbon of the labeled citrate that is retained in the rat is converted to $C^{14}O_2$ in 3 hours. Approximately 1 per cent of the administered radioactivity was recovered in protein and lipid in the kidneys and liver. The incorporation of C^{14} into the kidney tissue fractions was greater than that into the corresponding liver fractions.

In vivo and *in vitro* experiments demonstrated a divergent action of fluoroacetate and fluorocitrate as inhibitors of metabolism. In the intact animal fluoroacetate treatment inhibited the urinary excretion of administered citrate-C^{14} and enhanced its conversion to respiratory $C^{14}O_2$; fluorocitrate treatment resulted in an augmentation of the urinary excretion of the citrate-C^{14} and depressed its conversion to respiratory $C^{14}O_2$. Studies of the metabolism of liver and kidney slices in the presence of fluoroacetate or fluorocitrate indicated a difference in the mechanism of action of these inhibitors.

Although the distribution of citrate in the subcellular fractions of liver differed from that found in kidney after fluoroinhibitor administration, the localization in each of the tissues was the same, regardless of whether the rat received fluoroacetate or fluorocitrate.

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THE DETERMINATION OF UNEVEN PULMONARY BLOOD FLOW FROM THE ARTERIAL OXYGEN TENSION DURING NITROGEN WASHOUT

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Many tests are available for measuring unevenness of pulmonary ventilation. In recent years tests of unevenness of pulmonary blood flow have been described, which utilize radioactive krypton (1) in patients with emphysema, radioactive oxygen in patients with mitral stenosis (2), and radioactive carbon dioxide in normal subjects (3). These studies provide quantitative data on the degree of unevenness of pulmonary blood flow but require expensive and complicated apparatus and are not easily adapted to clinical pulmonary function testing. Simpler methods of estimating unevenness of blood flow in relation to ventilation have been suggested. These are based on the rate of rise of arterial oxygen saturation (ear oximeter) during the inhalation of oxygen (4–6), analysis of gas samples obtained from various lobes of the lungs (7), continuous analysis of a single expiration (8), and the difference between the nitrogen tension of alveolar gas and urine (9, 10). These simpler methods do not provide quantitative data on the degree of unevenness of pulmonary blood flow.

An ingenious graphical method of determining unevenness of perfusion of the lung in patients with chronic pulmonary emphysema has been worked out by Briscoe and co-workers (11). This method utilizes a nitrogen washout and wash-in technique and the arterial saturation during air-breathing. Its disadvantage is that other causes of arterial desaturation—i.e., shunts and diffusion barriers—cause an overestimation of the degree of unevenness of perfusion.

In this laboratory we have devised a test which yields quantitative data for the determination of the fractional alveolar ventilation and pulmonary capillary blood flow that go to the well and poorly ventilated regions of the lungs. The test requires the use of a nitrogen meter and a flow-through cuvet O_2 electrode for measuring arterial P_{O_2} continuously during the inhalation of O_2. In practice the test takes approximately 20 minutes and the calculations another 20 minutes. This method is based on the principle that the rate of rise of alveolar O_2 tension during oxygen inhalation depends on the distribution of inspired O_2 to well and poorly ventilated regions of the lung (in relation to their volume), while the rate of simultaneous rise of arterial O_2 tension depends on the distribution of pulmonary blood flow to these regions and the anatomical shunts. The theory, method and results of these studies in normal subjects and in patients with cardiac and pulmonary disease are presented here.

THEORY

The lungs can be divided into a well ventilated region (which need not be homogeneous) and a poorly ventilated region (subscripts 1 and 2, respectively). Arterial blood is a mixture of blood that has passed through these regions or through venous-to-arterial shunts. Its oxygen content (C_a) is related to the blood flows through the capillaries of the various regions ($\dot{Q}_1$, $\dot{Q}_2$) and through the shunt ($\dot{Q}_s$), and to their respective oxygen contents (C_1, C_2 and $C_{\bar{v}}$) as follows:

$$C_a \cdot \dot{Q}_T = C_1 \cdot \dot{Q}_1 + C_2\dot{Q}_2 + C_v\dot{Q}_s \qquad [1]$$

where $\dot{Q}_1 + \dot{Q}_2 + \dot{Q}_s = \dot{Q}_T$, the cardiac output. In this and subsequent formulations we omit, from our otherwise conventional (12) symbolization, the subscript c for capillary and the chemical subscript O_2.

It follows from Equation 1 that at any time (t) during the N_2 washout:

$$Ca_t = C_{1t}\left(\frac{\dot{Q}_1}{\dot{Q}_1 + \dot{Q}_2}\right) + C_{2t}\left(\frac{\dot{Q}_2}{\dot{Q}_1 + \dot{Q}_2}\right) - (C_a - C_v)_t\left(\frac{\dot{Q}_s}{\dot{Q}_1 + \dot{Q}_2}\right) \qquad [2]$$

We assume the following are constant in the later stages of the N_2 washout: *1*) the magnitude of the various blood flows ($\dot{Q}_1$, $\dot{Q}_2$, $\dot{Q}_s$, $\dot{Q}_T$) relative to each other; *2*) the arteriovenous O_2 content difference ($C_a - C_{\bar{v}}$). When we then

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consider the difference between the final equilibrium state at time f and the changing state at time t, it follows that:

$$Ca_f - Ca_t = (C_{1f} - C_{1t})\left(\frac{\dot{Q}_1}{\dot{Q}_1 + \dot{Q}_2}\right) + (C_{2f} - C_{2t})\left(\frac{\dot{Q}_2}{\dot{Q}_1 + \dot{Q}_2}\right) \quad [3]$$

Late in the washout when hemoglobin is fully saturated in all parts of the lung, further changes in O_2 content are linearly related to changes in O_2 tension (13). Equation 3 can then be formulated in terms of differences in P_{O_2}:

$$Pa_f - Pa_t = (P_{1f} - P_{1t})\left(\frac{\dot{Q}_1}{\dot{Q}_1 + \dot{Q}_2}\right) + (P_{2f} - P_{2t})\left(\frac{\dot{Q}_2}{\dot{Q}_1 + \dot{Q}_2}\right) \quad [4]$$

Any P_{O_2} gradient due to diffusion difficulty becomes infinitesimal at this time when P_{O_2} is high in all alveoli (13–15). Alveolar and end-capillary oxygen tensions are thus identical within a region; i.e., $P_{A_1} = P_1$, $P_{A_2} = P_2$. If P_{CO_2} is constant within each region, then P_{O_2} is related to P_{N_2}, provided that the total pressure of all gases is constant, as it is, in each region, and hemoglobin is fully saturated, as it is, at this time. Then:

$$Pa_f - Pa_t = \Delta P_{1N_2}\left(\frac{\dot{Q}_1}{\dot{Q}_1 + \dot{Q}_2}\right) + \Delta P_{2N_2}\left(\frac{\dot{Q}_2}{\dot{Q}_1 + \dot{Q}_2}\right) = \Delta P\bar{c}_{N_2} \quad [5]$$

where the various values of ΔP_{N_2} are the excess at time t over the final[1] value at time f. $\Delta P\bar{c}_{N_2}$ is the mean value of this excess in mixed end-capillary blood. This relation holds, regardless of the number of regions.

Late in the washout the concentration of nitrogen has become negligible in all but the worst ventilated region. The contributions of N_2 from the inspired O_2 tank and from the tissues are virtually constant; while they affect the final value they are of no significance in these considerations of the changes in P_{N_2}. It follows that, late in the washout: $\dot{V}_{AT} \cdot \Delta P_{AN_2} = \dot{V}_{A_2} \cdot \Delta P_{A_2N_2}$ and $\dot{Q}_T \cdot \Delta P_{aN_2} = \dot{Q}_2 \cdot \Delta P_{2N_2}$, where the subscript T refers to total alveolar ventilation or total cardiac output. Since $\Delta P_{A_2N_2} = \Delta P_{2N_2}$, these two equations yield the ratio between those fractions of total alveolar ventilation and total perfusion, respectively, which are distributed to the poorly ventilated region:

$$(\dot{V}_{A_2}/\dot{V}_{AT})/(\dot{Q}_2/\dot{Q}_T) = \Delta P_{AN_2}/\Delta P\bar{c}_{N_2} \quad [6]$$

At the end of the N_2 washout this ratio is constant, since semilogarithmically plotted values of $\Delta P\bar{a}_{N_2}$ and $\Delta P\bar{c}_{N_2}$ are found to lie on parallel lines. When $(\dot{V}_{A_2}/\dot{V}_{AT})$, $\Delta P\bar{a}_{N_2}$ and $\Delta P\bar{c}_{N_2}$ are experimentally determined, $\dot{Q}_2/\dot{Q}_T$ can be calculated. No attempt has been made to estimate the distribution of perfusion to compartments in the well ventilated region, because the assumptions that allow the calculation of $P\bar{c}_{N_2}$ are not valid in the early portion of the N_2 washout. The distribution of pulmonary blood flow is matched with the distribution of alveolar ventilation when the fractional perfusion equals the fractional ventilation of the poorly ventilated region.

The dilution index. A template was made in which $\log \{1/[1 + (1/n)]\}^n$ is plotted against n. This was laid over the semilogarithmically plotted data and analyzed into linear components (16, 17) (Figure 1). The intersection of the curved template with each straight line gives a value of n (on the baseline) which is the dilution index. This index, which is related to the turnover rate (k) of Robertson, Siri and Jones (16) is the ratio FRC/VT − VD for each region.

The use of the dilution index and its determination by a template has proved to be a useful shortcut in the calculation of the functional residual capacity (FRC) and alveolar tidal volumes of the well and poorly ventilated regions. It also provides a simple characterization of the alveolar ventilation of any region.

Mixed expired alveolar versus end-tidal nitrogen concentrations. In our study we measured end-tidal N_2 concen-

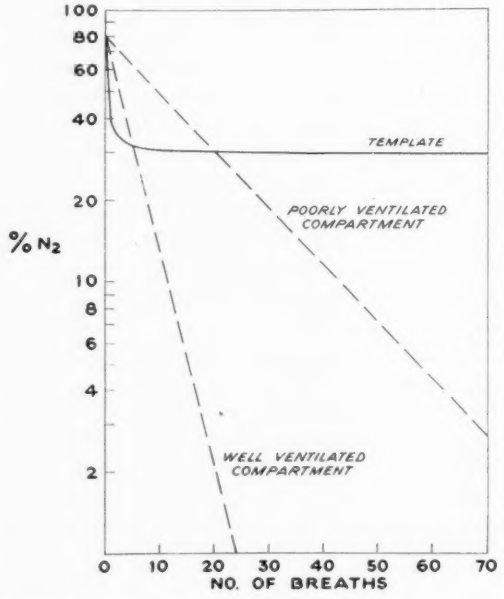

FIG. 1. PERCENTAGE OF N_2 CONCENTRATION IN END-EXPIRED GAS ON LOGARITHMIC SCALE PLOTTED AGAINST NUMBER OF BREATHS FOR THE WELL AND POORLY VENTILATED REGIONS OF THE LUNG. The dotted lines represent the N_2 washouts of the well and poorly ventilated compartments obtained from the N_2 end-tidal washout. The dilution index template is superimposed on these graphs, giving an intercept value with the well ventilated region at breath 5 and with the poorly ventilated region at breath 20.

[1] For present purposes we consider N_2 washout complete when the forced end-expired P_{N_2} is less than 4 mm Hg.

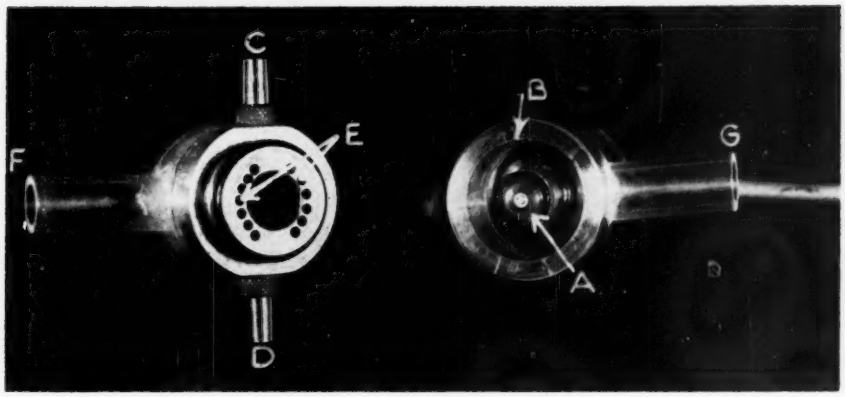

FIG. 2. RIGHT, CLARK O_2 ELECTRODE (A) IN LUCITE JACKET (B) AND LEFT, THE FLOW-THROUGH CUVET (C-D) FOR THE ELECTRODE SHOWING THE HOLES (E) THAT ALLOW CIRCULATION THROUGH TUBES (F AND G) FROM THE WATERBATH AROUND CUVET AND ELECTRODE. The left-hand part fits snugly beneath the right-hand part and the membrane of the Clark electrode is in the flowing blood stream.

tration. The fact that these concentrations exceed mixed alveolar and mixed expired concentrations does not affect our determination of the dilution indices n_1 and n_2 (18). But this fact does necessitate the use of an additional piece of information in determining the absolute volumes and ventilations of the various regions. We have used the following relationship to determine alveolar ventilation for the less ventilated alveoli $(V_T - V_D)_2$:

$$(V_T - V_D)_2 = \frac{FRC_T - n_1(V_T - V_D)}{n_1 - n_2} \qquad [7]$$

Where FRC_T, V_T, and V_D are, respectively, the total FRC, tidal volume, and dead space.

METHODS AND MATERIAL

The seated subject breathed from a system which delivered air or oxygen on demand. Inspired and expired gas were sampled continuously in the mouthpiece, and passed through an infrared CO_2 meter and a N_2 meter, both calibrated before and after each study with gases analyzed with the Scholander gas analyzer. While the subject breathed air, expired gas was collected for 3 minutes in a Neoprene metereological balloon. A sample was taken for O_2 and CO_2 analysis and the volume measured with a Tissot spirometer. Brachial arterial blood was sampled through an indwelling polyethylene catheter. Its P_{CO_2} was measured with the Severinghaus CO_2 electrode (19). During N_2 washout studies arterial O_2 tension was measured continuously with a Clark electrode placed in a temperature-controlled cuvet (Figure 2), through which blood flowed at a constant rate in a range of 5 to 10 ml per minute. The data were recorded on a Grass polygraph (see Figures 3, 5 and 7). Barometric pressure was measured at each study and was used to calculate all tensions.

This flow-through O_2 electrode was calibrated by analyzing samples of arterial blood at the beginning and the end of the N_2 washout on a Severinghaus modification of the Clark O_2 electrode (19). The Clark electrodes used in this study were slightly alinear over the 0 to 95 per cent O_2 range. This alinearity occurred below 21 per cent O_2, which affected the accuracy of the P_{O_2} record in the earlier part of each study but not in the later part used in determining the distribution of perfusion. The final Pa_{O_2} used in each case was the constant highest value, regardless of the time required to attain it.

The reproducibility of the method was checked in dogs in which the arterial blood from the femoral artery that passed through the cuvet was returned continuously by vein. In 3 dogs studied this way repeated N_2 washouts on a constant volume ventilator, and continuous recordings of Pa_{O_2} were identical for the individual dogs.

The arterial P_{O_2} tracing lagged behind the tracings recorded at the mouth for two reasons. The first was the circulation time from the alveolar capillaries to the cuvet, which averaged about 12 seconds; this was measured as the time between the rise in alveolar O_2 tension occurring during the first inhalation of O_2 and the onset of the rise in arterial P_{O_2}. The second was the slow response time of the electrode; the mean time for 90 per cent response after a square wave rise in P_{O_2} in either blood or gas was 9 seconds. Because the rate of rise of the arterial O_2 tension was not constant during the N_2 washout, it was necessary to correct the rate of rise in O_2 tension for the electrode lag. The over-all lag was checked by having the patient perform a Valsalva maneuver for several seconds at the completion of the N_2 washout. This maneuver causes a square-wave rise in O_2 tension in the alveolar gas almost equal to the total rise in alveolar pressure. The time for a new plateau of arterial O_2 tension to occur was approximately the same as the sum of the circulation time and time for 90 per cent electrode response, determined separately.

In addition to these studies, the following pulmonary function tests were performed: 7-minute N_2 washout (20);

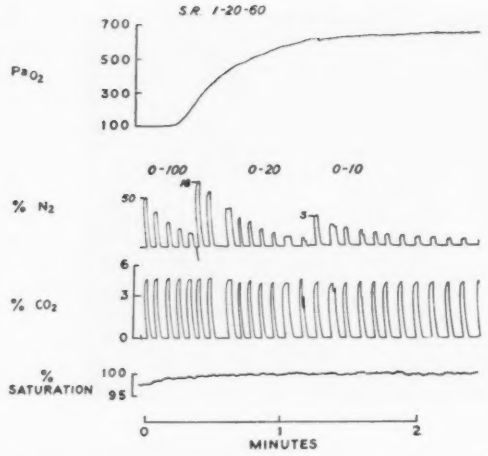

FIG. 3. CONTINUOUS RECORDING OF ARTERIAL O_2 TENSION, END-TIDAL N_2 AND CO_2 CONCENTRATIONS AND EAR OXIMETER. Normal subject; inhalation of O_2 beginning at 0 minutes.

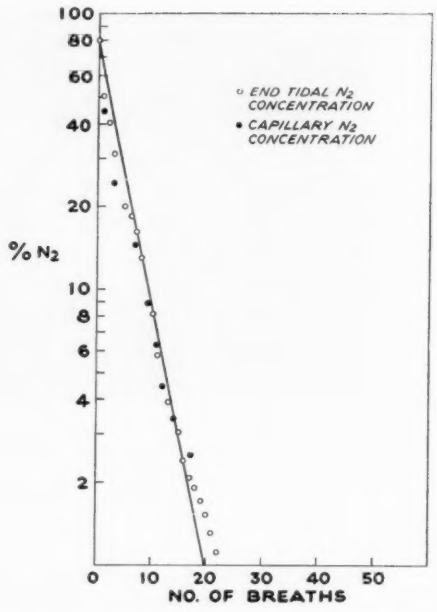

FIG. 4. PER CENT CONCENTRATION OF END-TIDAL AND CAPILLARY N_2 PLOTTED ON LOGARITHMIC SCALE AGAINST NUMBER OF BREATHS FOR THE SAME SUBJECT OF FIGURE 3.

FRC at the end of 7 minutes (20); single breath O_2 test of distribution of inspired air (21); calculation of physiological dead space (22); maximal inspiratory and expiratory flow rates; and, on patients with interstitial fibrosis, pulmonary diffusing capacity (23). Anatomic dead space was estimated to be 30 per cent of the tidal volume. This value was chosen, since it represented the average PDS/VT × 100 in the normal subjects whose VT ranged from 420 to 970 ml.

RESULTS

Normal subjects. Figure 3 shows a typical recording. Both the N_2 washout and the rise of arterial O_2 tension are fast and "complete" in 20 breaths. Figure 4 shows the semilogarithmic plot of per cent N_2 in end-tidal gas and capillary blood. The data in Table II show that the $\dot{V}_A/\dot{Q}_c$ ratios are approximately the same in both the well and poorly ventilated regions despite the fact that the well ventilated region has considerably more ventilation per unit alveolar volume than has the poorly ventilated compartment. The two plots are superimposable.

Patients with pulmonary emphysema. Figure 5 shows that both the rise in arterial O_2 tension

TABLE I

Results of pulmonary function tests (mean and range)

Groups	Subjects	Age	FRC	S.B.O_2*	7-min N_2 washout	PDS/VT	P_{CO_2} (a-A)	$P_{a_{O_2}}$ (air)	$P_{a_{O_2}}$ (O_2)	Dco
	no.	*yrs*	*L*	*% N_2*	*% N_2*	*ml/ml*	*mm Hg*	*mm Hg*	*mm Hg*	*% pred.*
Normal	9	31	3.1	0.7	0.5	0.30	1.5	95	645	115
		21–38	2.2–3.9	0.0–2.4	0.5–0.7	0.27–0.35	1.0–4.0	85–105	610–660	102–125
Emphysema	15	59	4.4	7.2	6.9	0.51	7.0	70	585	
		44–76	3.1–6.2	2.1–13.0	2.3–17.2	0.33–0.67	2.0–13.0	60–90	460–640	
Interstitial fibrosis	7	45	2.1	4.7	1.4	0.43	3.5	80	615	49
		23–53	1.3–3.2	1.0–11.0	1.0–2.2	0.33–0.59	0.0–8.0	65–90	565–660	23–67
Mitral valv. dis. + pulm. hypertens.	4	43	2.2	5.0	2.3	0.44	6.0	75	600	
		29–49	1.0–3.0	4.0–6.0	0.6–3.4	0.37–0.55	4.0–9.0	55–80	580–615	

* Single-breath oxygen test.

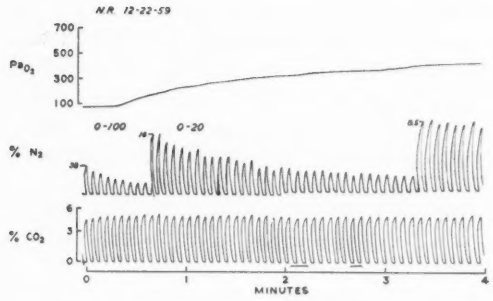

FIG. 5. PATIENT WITH EMPHYSEMA; CONTINUOUS RECORDING OF ARTERIAL BLOOD O_2 TENSION, END-EXPIRED N_2 AND CO_2 CONCENTRATIONS DURING INHALATION OF O_2.

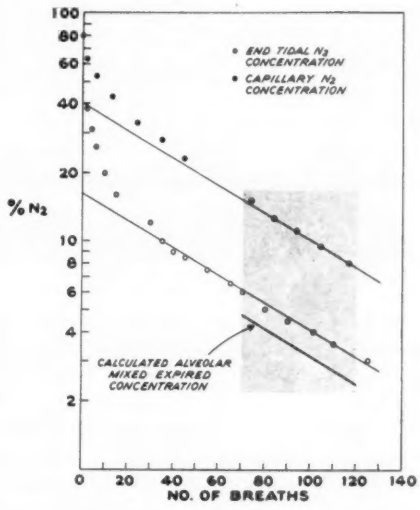

FIG. 6. PER CENT CONCENTRATION OF END-TIDAL AND CAPILLARY N_2 PLOTTED ON LOGARITHMIC SCALE AGAINST NUMBER OF BREATHS FOR THE PATIENT SHOWN IN FIGURE 5. The shaded area represents the portion of washout where calculation of distribution of ventilation, perfusion and mean expired alveolar P_{N_2} was performed.

and the fall in alveolar N_2 concentration are delayed. Although the delay is greater for the blood curve, this can best be determined by plotting semilogarithmically per cent N_2 in end-tidal gas and capillary blood (Figure 6). There is a marked difference between the two, owing to overperfusion or underventilation of the poorly ventilated space. The shaded area represents the region selected for the calculation of ventilation and perfusion distribution to the poorly ventilated region. Table II shows that there is a marked difference in ventilation-perfusion ratios of the two regions in patients with emphysema. The percentage of the total ventilation distributed to the two regions is similar to that found in the normal subjects, but there is a marked increase in the volume of the poorly ventilated region so that the ventilation of this space is markedly reduced in relation to volume. In addition, there is a marked increase in perfusion through the poorly ventilated region in terms of percentage of total pulmonary blood flow; in relation to volume, this perfusion is much the same as in the normal group. The reduced $V_A/\dot{Q}_c$ ratio in the poorly ventilated space is reflected in the low arterial O_2 tension when these patients breathe air and in the increase in PDS/VT ratio and arterial end-tidal P_{CO_2} differences.

Patients with mitral valvular disease and pulmonary hypertension. Two subjects had pure

TABLE II

Distribution of functional residual capacity, ventilation, perfusion and the fractional ventilation to perfusion ratios of the well ventilated and poorly ventilated regions (mean and range) in normal subjects and patients with pulmonary and cardiac disease

Groups, no.	Well ventilated region				Poorly ventilated region			
	FRC%	$\dot{V}_A$%	$\dot{Q}_c$%	$\dot{V}_A$%/$\dot{Q}_c$%	*FRC%*	$\dot{V}_A$%	$\dot{Q}_c$%	$\dot{V}_A$%/$\dot{Q}_c$%
Normal, 9	60 14–100	82 58–100	81 50–100	1.03 1.00–1.16	40 0–86	18 0–42	19 0–50	0.92 0.84–1.00
Emphysema, 15	32 8–69	85 74–95	49 34–63	1.78 1.35–2.42	68 31–92	15 5–26	51 37–66	0.30 0.11–0.56
Interstitial fibrosis, 7	39 8–89	81 54–94	59 15–87	1.65 1.05–3.60	61 11–92	19 6–46	41 13–85	0.51 0.14–0.91
Mitral valv. dis. + pulm. hypertens., 4	41 39–45	83 75–90	44 22–67	2.36 1.35–3.64	59 55–61	17 10–25	56 33–78	0.31 0.26–0.33

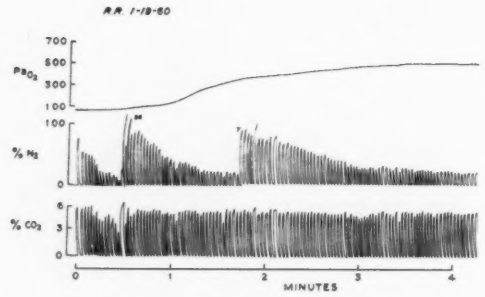

FIG. 7. PATIENT WITH MITRAL STENOSIS; CONTINUOUS RECORDING OF ARTERIAL BLOOD O_2 TENSION, END-TIDAL N_2 AND CO_2 CONCENTRATIONS DURING INHALATION OF O_2.

mitral stenosis, and two had mitral stenosis and insufficiency. All had pulmonary hypertension confirmed by pressure recordings during cardiac catheterization. A record from a patient with pure mitral stenosis and a mean pulmonary arterial pressure of 50 mm Hg is shown in Figure 7. There is a very slow rise in arterial O_2 tension. Tables I and II show unequal ventilation-perfusion ratios similar to those found in the patients with emphysema. These findings were reflected in the increased PDS/VT ratios, the increased P_{CO_2} differences between arterial blood and alveolar gas.

DISCUSSION

In developing the equations that allow calculation of end-capillary N_2 tension, several assumptions were made. The first was that the pulmonary blood flow reached a fixed distribution pattern late in the N_2 washout; i.e., the fractional blood flow through the well and poorly ventilated region became constant. This merely implies that any changes due to O_2 were proceeding at a negligible rate near the end of the O_2 breathing period when our determinations were made. The perfusion distribution calculated by this method is that which occurs during pure ogyxen breathing.

The second assumption is that the right-to-left anatomical shunt becomes a fixed fraction of the pulmonary blood flow during N_2 washout. In normal subjects the anatomical shunt becomes constant when alveolar P_{O_2} is raised stepwise (16).

The third assumption is that the arteriovenous O_2 content difference (C_a–$C_{\bar{v}}$) reaches a constant value over the latter part of the washout. There is no reason why this should change at a time when the subject is virtually in a steady state.

The fourth assumption, that diffusion barriers present no impediment when $P_{A_{O_2}}$ is high, is valid and has already been discussed under Theory.

The fifth assumption, that the P_{CO_2} is constant, though at various values in different regions, is valid in a steady state such as this.

The last assumption is that the lung can be divided into only a well and a poorly ventilated region. This assumption permits the calculation of mixed alveolar P_{N_2} from the end-tidal P_{N_2} graph. In order to check the reasonableness of this assumption, a model lung with a distribution of ventilatory regions varying over a wide range was constructed mathematically. When this model was subjected to a N_2 washout, and the alveolar P_{N_2} was plotted on two-cycle semilogarithmic paper, only two regions could be found by the graphical technique (24). The two-ventilatory-region simplification is practical as well as convenient. The use of the 7-minute FRC to calculate the fractional ventilation to the poorly ventilated region is convenient but, in patients with chronic pulmonary emphysema, introduces a small inaccuracy. A recent study (25) has shown that the conventional 7-minute FRC measured by the N_2 washout method underestimates the prolonged N_2 washout FRC by 19 to 48 per cent in patients with chronic pulmonary emphysema. In this laboratory the 7-minute FRC measured by the N_2 open-circuit and the closed-circuit methods underestimated the 18-minute FRC by an average of 980 ml in 13 patients with chronic pulmonary emphysema (26). Using the 18-minute rather than the 7-minute FRC value increases the ventilation to the poorly ventilated region approximately 20 per cent, or an increase from 15 to 18 per cent in the value $\dot{V}_A$ per cent to the poorly ventilated region in Table II.

The contributions of N_2 from the inspired O_2 tank and from the tissues have a virtually constant effect and so do not influence the various values of ΔP_{N_2} used here, which are differences from the final state. Hence the ratio $\Delta P\bar{A}_{N_2}/\Delta P\bar{c}_{N_2}$ is not affected.

Measurement of arterial P_{O_2} during the N_2

washout can be made continuously, as here, or by drawing individual samples at different times during the washout. The accuracy of $P_{a_{O_2}}$ measurements in the high P_{O_2} range is ± 5 mm Hg. The advantage of the directly recorded $P_{a_{O_2}}$ is that $P_{a_{O_2 y}} - P_{a_{O_2 t}}$ can be graphed over the entire N_2 washout and the proper range can be selected late in the washout when the slopes of $P\bar{A}_{N_2}$ and $P\bar{c}_{N_2}$ are parallel.

The results show that in normal subjects the ratio of ventilation to perfusion is approximately the same for both the well and the poorly ventilated regions. This finding is in agreement with the results obtained by other methods (3) and is to be expected from the normal values for pulmonary function obtained in these subjects.

In the patients with emphysema, there was a consistently lower ventilation-perfusion ratio in the poorly ventilated region. The perfusion of the two regions in the patients with emphysema was more closely related to the volume of the region than to its ventilation. This confirms work recently published by Briscoe and associates (11). This can be interpreted in two ways. One is that the poorly ventilated region represents the more normal lung tissue which is still normally perfused, while the well ventilated area represents tissue with increased compliance and ventilation in which there has been loss of capillary bed. Another is that the reverse situation exists and the poorly ventilated region represents the more diseased areas in which ventilation is markedly inadequate and perfusion is still relatively in excess of the inadequate ventilation. Further studies are needed to establish correlations between the physiological findings such as ours and the anatomical distribution of the alveolar and capillary lesions in patients with emphysema.

The results in patients with fibrosis showed great variation and are difficult to interpret. In some patients the ventilation-perfusion relationships appeared relatively normal, while in others the findings were similar to those in patients with emphysema. These findings are consistent with those of others (27, 28).

In the patients with mitral disease and pulmonary hypertension, there were abnormalities of ventilation-perfusion relationships similar to those seen in patients with emphysema. It has been shown (29) that the distribution of the pulmonary vascular lesions in such patients is not uniform. The greatest medial hypertrophy and intimal thickening is found in the bases, while the apical regions of the lungs are relatively normal. It is possible that the abnormalities in $\dot{V}_A/\dot{Q}_c$ in our patients with mitral stenosis and pulmonary hypertension are due to these differences in the pulmonary vasculature.

SUMMARY

1. A new method is described for obtaining quantitative data on distribution of pulmonary capillary blood flow through well and poorly ventilated regions of the lungs. It requires continuous or repeated analyses of alveolar P_{N_2} and arterial P_{O_2} during inhalation of O_2.

2. End-capillary P_{N_2} is calculated from the arterial O_2 tension during the N_2 washout.

3. The ratio of $P\bar{A}_{N_2}$ to $P\bar{c}_{N_2}$ late in the washout gives the ratio of fractional ventilation to fractional perfusion through the poorly ventilated space.

4. Unevenness of perfusion exists when the fractional perfusion is in excess of fractional ventilation to the poorly ventilated space.

5. Normal subjects have no unevenness of perfusion in relation to ventilation.

6. Patients with emphysema have marked unevenness of perfusion in relation to ventilation but not in relation to lung volume.

7. Patients with interstitial fibrosis may have unevenness of perfusion in relation to ventilation.

8. Patients with mitral valvular disease and pulmonary hypertension have unevenness of perfusion in relation to ventilation.

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THE EFFECT OF PHENYLHYDRAZINE ON THE ADENOSINE TRIPHOSPHATE CONTENT OF NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT HUMAN BLOOD *

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The erythrocytes of certain individuals are peculiarly sensitive to the hemolytic effects of primaquine and a number of related compounds. A variety of biochemical abnormalities has been uncovered in such erythrocytes, principally related to deficiency of glucose-6-phosphate dehydrogenase (G6PD) (1–3) but the mechanisms responsible for the drug-induced hemolysis have not been clearly defined.

Adenosine triphosphate (ATP) is an important compound in the metabolism of the erythrocyte, and it is known that a decrease in the concentration of ATP in erythrocytes correlates well with decreased survival of these cells as they age *in vitro* (4, 5) and *in vivo* (6, 7). It therefore seems possible that a fall in the level of ATP might be associated with the decreased erythrocyte survival that occurs in drug-induced hemolytic anemia. In order to obtain evidence bearing on this point phenylhydrazine hemolysis was chosen as a model. The effect of phenylhydrazine on the ATP of whole blood from normal subjects and from individuals with G6PD deficiency was determined under a variety of *in vitro* experimental conditions. Herein are presented data from experiments which demonstrate that phenylhydrazine induces a marked fall in the ATP level of both normal and G6PD-deficient blood. The fall in ATP can be partially prevented by glucose in both types of blood, but the protective effect is less in G6PD-deficient blood.

MATERIALS AND METHODS

Subjects studied. All subjects were adult males. The G6PD-deficient subjects were all Negroes who had normal hemoglobin by electrophoretic analysis performed on starch blocks (8). In all subjects the leukocyte count was normal, the hematocrit above 40 per cent, and the reticulocyte count below 3.3 per cent. The hematologic studies were performed by standard methods (9). G6PD was assayed by following the appearance of reduced triphosphopyridine nucleotide (TPNH) spectrophotometrically under the conditions described by Motulsky (10): The reaction mixture contained 3 μmoles disodium ethylenediamine tetraacetic acid; 20 μmoles $MgCl_2$; 39.5 μmoles nicotinamide; 222 μmoles Tris-(hydroxymethyl) aminomethane; 5 μmoles glucose-6-phosphate; 0.4 mg triphosphopyridine nucleotide (TPN); and 2 ml of a 1:250 aqueous hemolysate of whole blood in a final volume of 3 ml. The reaction was carried out at room temperature in cuvets of 1 cm light path in a Beckman model DU spectrophotometer. Units of activity are defined as change in optical density at 340 mμ per minute per ml of erythrocytes.

Preparation of blood. Whole blood was collected in heparin (0.1 mg per ml of blood) under sterile conditions from fasting subjects. Whole blood was used for most experiments to keep manipulation of the erythrocytes to a minimum. The data of Bishop, Rankine, and Talbott (11) and of Whittam (12) indicate that essentially all the ATP of whole blood is in the erythrocytes, and in the present study no significant difference was found between the ATP content of whole blood and an equivalent amount of washed erythrocytes from a suspension which had a hematocrit of 35 per cent and contained 2,000 platelets and 1,850 leukocytes per mm^3.

Washed erythrocytes were prepared at 4° C as described by Whittam (12) except that balanced salt solution buffered with bicarbonate was used throughout. The erythrocytes were finally suspended in sufficient balanced salt solution to restore the volume to that of the original blood.

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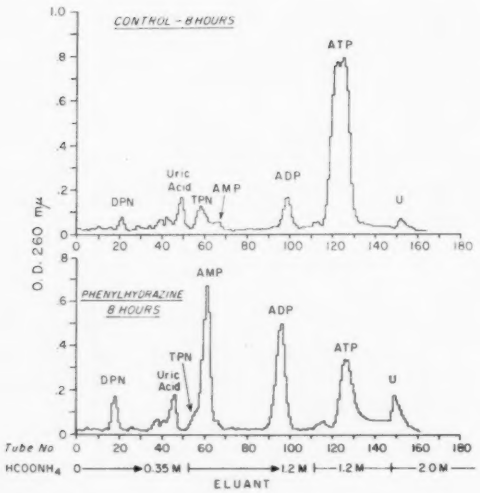

FIG. 1. CHROMATOGRAM OF NUCLEOTIDES EXTRACTED FROM WHOLE BLOOD INCUBATED WITH AND WITHOUT PHENYLHYDRAZINE, SHOWING RISE IN AMP AND ADP AND FALL IN ATP LEVEL INDUCED BY PHENYLHYDRAZINE. The chromatograms were obtained from TCA extracts of 10 ml whole blood incubated for 8 hours at 37° C with and without phenylhydrazine, as outlined under Methods. Chromatography was carried out on 1×20 cm columns of Dowex 1×8 formate resin, 200–400 mesh. The flow rate was 0.5 to 0.8 ml per minute; 6-ml fractions were collected. The gradient elution system used achieved a concave gradient by means of a conical reservoir containing 140 ml solvent and a cylindrical mixing vessel containing 220 ml solvent. Solutions of ammonium formate at pH 4.5 were used as eluant. The first gradient was from 0 to 0.35 M ammonium formate and the second from 0.35 to 1.2 M. After this the chromatogram was completed by straight elution with 1.2 M and 2.0 M ammonium formate. Abbreviations used are: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; and U, unknown components.

Incubation of blood. Ten ml of blood or washed erythrocyte suspension was placed under sterile conditions in silicone-coated (G. E. Drifilm) screw-capped tubes. Phenylhydrazine hydrochloride dissolved in 0.9 per cent sodium chloride solution was sterilized by filtration and was added to the appropriate vessels to a final concentration of 3.4 μmoles per ml. Sterile glucose solution in 0.9 per cent sodium chloride was added to the appropriate vessels to give a final concentration of added glucose of 20 μmoles per ml. All reaction mixtures were made to a final volume of 11.0 ml with 0.9 per cent sodium chloride solution when necesary. Incubation was carried out at 37° C in an atmosphere of air with intermittent inversion of the tubes to maintain suspension of the cells.

At the end of each period of incubation the pH, per cent hemolysis (13), and Heinz body formation (14) were determined on a 1.0 ml aliquot of the reaction mixture, and a cold trichloroacetic acid (TCA) extract was made from the remainder by the method of Bishop and co-workers (11). Glucose was determined at intervals throughout the incubation period by a glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N. J.). Pyruvic acid was measured by the method of Bueding and Wortis (15) and lactic acid by the method of Barker and Summerson (16).

Column chromatography. The acid-soluble nucleotides were chromatographed on 1×20 cm columns of Dowex 1×8 formate resin (200–400 mesh) (17). After the extract had passed through the resin, the column was washed with water until the effluent no longer absorbed ultraviolet light. Elution of the nucleotides was then carried out with solutions of ammonium formate at pH 4.5, with a flow rate of 0.5 to 0.8 ml per minute. Separation of all the major nucleotides was achieved with a concave gradient system (18) employing a conical reservoir and a cylindrical mixing vessel (19). The details of the technique are given in Figure 1.

In order to simplify the estimation of ATP, a chromatographic system was developed which separated the nucleotides into a pre-ATP fraction, ATP, and a post-ATP fraction. The chromatogram was developed with 1.1 M ammonium formate at pH 4.5 until ATP was recovered, and then with 2.0 M ammonium formate at pH 4.5 to recover the post-ATP fraction. A chromatogram of this type is illustrated in Figure 2.

Fractions of 6 ml were collected for both types of chromatogram with an automatic fraction collector. The optical density at 260 and 275 mμ was determined for each fraction. Nucleotides were identified by their ultraviolet absorption spectra and by their position on the chromatogram as compared with standard compounds (purchased from Sigma Chemical Co., St. Louis, Mo.).

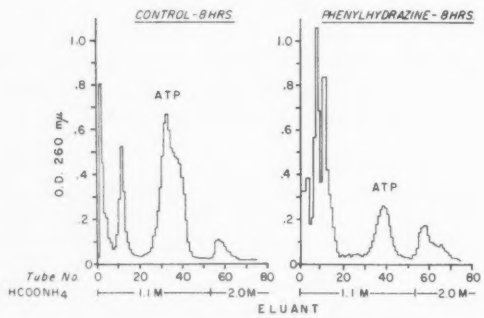

FIG. 2. SIMPLIFIED CHROMATOGRAPHIC PROCEDURE FOR MEASURING ATP. Blood was incubated as described in Figure 1. The chromatographic procedure was that used in Figure 1 except that elution was performed with a nongradient system utilizing 1.1 M and 2.0 M ammonium formate at pH 4.5, as indicated on the figure.

TABLE I

Changes in the pH, per cent hemolysis, and Heinz body formation of whole blood incubated with phenylhydrazine

Time of incubation, hours	0		4		6		8	
	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine
pH	7.6	7.6	7.52	7.58	7.45	7.4	7.35	7.35
Hemolysis, %	0.8	1.2	1.3	1.0	0.8	1.8	1.0	1.9
RBC with 5 or more Heinz bodies, %	0	0	0	90	0	94	0	96

In some experiments the adenine nucleotides were analyzed for ribose (20) and phosphate (21) content, and DPN and TPN were further identified by the change in extinction coefficient at 340 mμ which occurs on reaction with cyanide (22). Recovery of ultraviolet-absorbing material was greater than 90 per cent in all experiments with both types of chromatography. Also, recovery of known amounts of an ATP standard was greater than 94 per cent with both chromatographic systems.

RESULTS

Effect of phenylhydrazine on the ATP of whole blood in vitro. Data from an experiment on the effect of incubation of whole blood with phenylhydrazine on the pH, per cent hemolysis, and Heinz body formation are shown in Table I. In no experiment was the final pH less than 7.3; hemolysis never exceeded 2 per cent during an 8 hour incubation; and in most experiments after 4 hours' incubation with phenylhydrazine, more than 90 per cent of the erythrocytes contained more than 5 Heinz bodies.

Illustrated in Figure 1 are typical chromatograms obtained from the TCA extracts of normal whole blood incubated for 8 hours with and without phenylhydrazine. The chromatogram of blood incubated for 8 hours without phenylhydrazine does not differ significantly from that found with blood at zero time, and the quantities of nucleotides recovered at both 0 and 8 hours agree well with the data of Bishop and co-workers (11). Two definite changes in the nucleotide pattern of blood incubated with phenylhydrazine can be detected: first, there is a marked decrease in ATP and a corresponding increase in adenosine diphosphate (ADP) and adenosine monophosphate (AMP); and second, there is an increase in the material eluted from the column after ATP. This material has not been identified but appeared, from a study of the ultraviolet absorption spectrum, to be a mixture of a guanine compound with an adenine-like compound. In these experiments the final concentration of phenylhydrazine was 3.4 μmoles per ml; higher concentrations, up to 6.8 μmoles, caused a greater fall in ATP; lower concentrations, down to 1.7 μmoles per ml, were less effective in this regard.

TABLE II

Reciprocal rise in AMP and ADP after the fall in ATP occurring after 8 hours' incubation of whole blood with phenylhydrazine *

Subject	AMP	ADP	ATP	AMP & ADP & ATP	Adenine nucleotide recovered, %†
D.M. (normal) Control	7.5	35.2	372.5	415.2	93.1
Phenylhyd.	127.9	125.6	133.2	386.7	
R.D. (G6PD-def.) Control	5.9	54.5	339.5	399.9	85.6
Phenylhyd.	94.6	126.2	121.7	342.5	

* Concentrations are given as μmoles/L whole blood. See text for abbreviations.
† In each experiment 100% recovery was assumed for the control vessels.

Quantitative data on the adenine nucleotides from this experiment and a similar one on blood from a G6PD-deficient subject are presented in Table II. Most of the ATP which disappeared was found as ADP and AMP in both experiments, indicating that the fall in ATP was due primarily to loss of terminal phosphate groups.

Figure 2 shows chromatograms from experiments to determine the time course of the decrease in ATP which occurs when blood is incubated with phenylhydrazine. The simplified chromatographic system was used. In extracts of blood incubated with phenylhydrazine, the total amount of ultraviolet-absorbing material remained constant, but there was an increase in material eluted from the column before and after ATP and a decrease in ATP. Quantitative data on the ATP level obtained from such experiments on three normal and one G6PD-deficient subject are presented in Figure 3. It can be seen that the ATP of blood incubated with phenylhydrazine decreases, beginning at about 6 hours, and by 8 hours has fallen to about 35 per cent of the original amount present. In blood incubated without phenylhydrazine the ATP remained nearly constant. The results were the same in blood from normal subjects and a subject with G6PD deficiency.

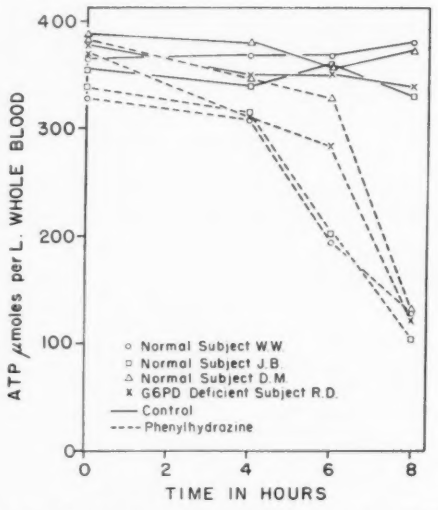

FIG. 3. RATE OF FALL OF ATP IN NORMAL AND G6PD-DEFICIENT BLOOD INCUBATED WITH AND WITHOUT PHENYLHYDRAZINE. ATP was determined on extracts prepared from 10 ml of blood incubated as outlined under Methods.

The effect of glucose on the ATP of phenylhydrazine-treated blood. The delay of several hours before the decrease in ATP of phenylhydrazine-treated blood suggested that the fall might be related to the depletion of glucose. Accordingly, experiments were carried out to assess the role of glucose in the changes in ATP in blood incubated with phenylhydrazine. Table III presents data from such experiments on three normal and three G6PD-deficient subjects. Glucose utilization averaged 0.66 μmole per ml of blood per hour for samples incubated without phenylhydrazine and 0.85 μmole per ml of blood per hour for samples incubated with phenylhydrazine. Blood from two normal and one G6PD-deficient subject was incubated at 37°

TABLE III

The effect of added glucose on the ATP content of normal and G6PD-deficient whole blood incubated with phenylhydrazine for 8 hours *

Subject	G6PD activity‡	No glucose added				% Decrease in ATP§	Glucose added†				% Decrease in ATP§
		Control		Phenylhydrazine			Control		Phenylhydrazine		
		ATP	Glucose	ATP	Glucose		ATP	Glucose	ATP	Glucose	
Normal											
W.W.	6.9	382.6	0.6	135.3	<0.1‖	64.6	389.4	19.3	343.4	16.6	11.8
B.F.	7.0	331.5	0.4	115.4	<0.1	65.2	341.1	22.9	297.6	21.7	12.8
E.B.	7.0	247.1	<0.1	79.2	<0.1	67.9	326.8	20.8	289.7	18.0	11.4
G6PD-def.											
R.D.	1.1	315.1	0.9	120.0	<0.1	61.9	348.6	22.0	224.8	20.5	35.5
L.J.	1.4	265.7	0.7	99.0	<0.1	62.7	287.4	23.4	172.7	21.2	39.9
J.C.	0.5	332.6	1.0	179.0	0.2	46.2	309.0	20.8	188.4	20.0	39.0

* ATP concentration expressed as μmoles/L blood, glucose concentration as μmoles/ml blood.
† The blood was supplemented with 20 μmoles per ml glucose.
‡ Glucose-6-phosphate dehydrogenase activity expressed as change in optical density/min/ml RBC.
§ Expressed as % fall in ATP content of phenylhydrazine-treated blood compared with control blood.
‖ Measurement of glucose not accurate below 0.1 μmole per ml.

C with or without phenylhydrazine (3.4 μmoles per ml) and with added glucose (20 μmoles per ml), and the amount of lactic and pyruvic acids formed was compared with the amount of glucose which disappeared. Although 88 to 103 per cent of the glucose utilized over an 8-hour period by both types of blood incubated with or without phenylhydrazine could be accounted for by lactic and pyruvic acids, phenylhydrazine altered the relative amounts of these two compounds formed. Thus, in both types of blood incubated without phenylhydrazine, pyruvic acid accounted for only 1 to 2 per cent of the glucose utilized, while in blood incubated with phenylhydrazine, 10 to 18 per cent of the glucose utilized appeared as pyruvic acid. These data indicate that glucose is metabolized principally by normal pathways in blood incubated with phenylhydrazine, although there appears to be interference with the reduction of pyruvic acid to lactic acid. The accumulation of pyruvic acid has been previously reported for phenylhydrazine-treated avian (25) and human (23) erythrocytes.

As shown in Table III, in blood incubated for 8 hours with phenylhydrazine but without added glucose, there was less than 0.1 μmole of glucose per ml remaining in all vessels except one (Subject J.C.). The ATP level was markedly reduced in all vessels compared with the controls, although in the blood from Subject J.C., where there was glucose still remaining after the incubation period, the fall was not so great as in the others. In the control vessels incubated for 8 hours without phenylhydrazine or added glucose, glucose remained in all vessels except one (Subject E.B.). The ATP level in the blood from Subject E.B. was lower than in the other control vessels but was still much higher than in the comparable vessel with phenylhydrazine added.

In the experiments on blood with glucose added, a large quantity of glucose remained after 8 hours' incubation with or without phenylhydrazine. In blood from normal subjects there was an average fall in ATP of 12 per cent, while in blood from G6PD-deficient subjects there was an average fall in ATP of 38 per cent. Thus, glucose partially protected G6PD-deficient erythrocytes and almost completely protected normal erythrocytes from the fall in ATP induced by phenylhydrazine after 8 hours' incubation.

TABLE IV

Normal whole blood incubated for 12 hours with phenylhydrazine and glucose added

Subject	Control		Phenylhydrazine		% Decrease in ATP*
	ATP	Glucose	ATP	Glucose	
	μmoles/L.	*μmoles/ml*	*μmoles/L.*	*μmoles/ml*	
D.M.	394.1	19.0	302.3	16.8	23.3
C.C.	323.5	18.0	202.9	17.5	37.3
B.F.	314.8	21.2	222.4	19.0	29.4

* Expressed as % fall in ATP content of phenylhydrazine-treated blood compared with control blood.

Prolonged incubation of normal blood with phenylhydrazine. Because there was a slight fall in ATP in normal blood incubated with phenylhydrazine and added glucose, additional experiments were performed to determine whether the protective effect of glucose on the ATP of normal blood would extend over a longer period of incubation. The results are shown in Table IV and demonstrate that, even with an adequate glucose concentration, incubation with phenylhydrazine for 12 hours causes a definite fall in the ATP content of normal blood.

Washed erythrocytes incubated with phenylhydrazine. Figure 4 illustrates the results of an experiment performed to determine the effect of phenylhydrazine on the ATP of erythrocytes which had been washed to remove most of the glucose. The washed erythrocyte suspension con-

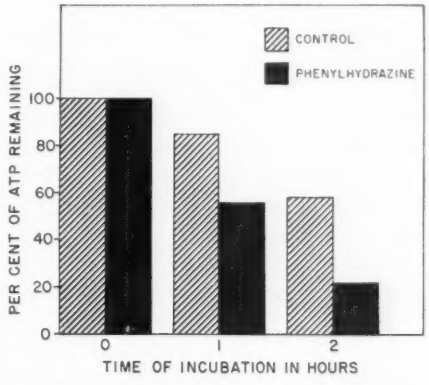

FIG. 4. THE EFFECT OF PHENYLHYDRAZINE ON THE ATP OF WASHED ERYTHROCYTES. The data are plotted as the per cent of zero time ATP remaining after each period of incubation. The hematocrit of the erythrocyte suspension was 36 per cent and the initial glucose concentration was 0.2 μmole per ml of suspension. The erythrocyte suspension was incubated as outlined under Methods.

tained 0.2 μmole of glucose per ml at the beginning of the incubation period. The fall in ATP level was more rapid in the samples incubated with phenylhydrazine than in the controls. The rate of disappearance of ATP from the control suspension was similar to that observed by Whittam (12).

DISCUSSION

The mechanism by which phenylhydrazine diminishes the ATP level of whole blood is at present unknown, although ultimately there must be either a decrease in ATP regeneration or an increase in ATP breakdown, or both. Phenylhydrazine is known to increase the rate of glucose utilization by blood (24) and, in those experiments in which blood was incubated with phenylhydrazine without added glucose, the fall in ATP might be accounted for in part by decreased regeneration of ATP as a result of exhaustion of glucose. In the experiments in which glucose was added to the incubation mixture in sufficient quantity to provide substrate throughout the experiment, a fall in ATP could still be demonstrated after 12 hours in normal blood and 8 hours in G6PD-deficient blood, which suggests that the effect of phenylhydrazine on the ATP level of blood cannot be ascribed entirely to increased glucose utilization. This point is further emphasized by the demonstration that a more rapid fall in ATP level occurred in washed erythrocytes incubated with phenylhydrazine than was found in the controls. Since essentially no glucose was available for ATP regeneration in these experiments, the results suggest that phenylhydrazine increases ATP breakdown instead of interfering with ATP regeneration. Further investigation will be necessary, however, to establish this point.

When phenylhydrazine is added to blood it reacts with oxyhemoglobin to form, among other products, hydrogen peroxide or equivalent free radicals (25–27). Hydrogen peroxide oxidizes hemoglobin (25–27) and other proteins (28, 29) including, perhaps, enzymes concerned with ATP metabolism. If hydrogen peroxide is the toxic agent responsible for the decline in ATP content of blood exposed to phenylhydrazine, then the difference in protective effect of glucose on the ATP of normal blood compared with G6PD-deficient blood may be tentatively explained on the following grounds. Hydrogen peroxide formed in the erythrocyte must be reduced by catalase or peroxidase, and we would expect the activity of both these enzymes to be suboptimal in G6PD-deficient blood incubated with phenylhydrazine. There are reports that catalase activity is decreased in the G6PD-deficient erythrocyte (30) and that phenylhydrazine is a catalase inhibitor (31). The "glutathione peroxidase" (32) of erythrocytes requires reduced glutathione (GSH) as a cofactor, and when hydrogen peroxide is reduced by this enzyme, oxidized glutathione (GSSG) is formed. GSSG must be continually reduced to GSH for peroxidase to remain active, and TPNH is the chief pyridine nucleotide necessary for this reduction (33). The G6PD-deficient cell is limited in its capacity to regenerate TPNH because metabolism of glucose via the pentose phosphate pathway, which is a source of TPNH, is interfered with by the enzyme deficiency. Hence, we would expect glucose to be less effective in protecting against the effects of hydrogen peroxide in the G6PD-deficient cell than in the normal cell, because the limiting factor would be the deficient enzyme.

The biochemical events leading to hemolysis in normal or G6PD-deficient erythrocytes exposed to phenylhydrazine or related compounds are still undefined. It seems possible that a decreasing level of ATP in the erythrocyte could lead eventually to hemolysis in view of the studies that have correlated a low level of ATP with impaired erythrocyte survival in stored blood (4, 5) and with aging of erythrocytes *in vivo* (6, 7). Other studies have shown that older erythrocytes (which have a low ATP content) (6, 7) are more susceptible to immune hemolysis (34), osmotic hemolysis (35, 36), and drug-induced hemolysis (37). The fall in ATP observed in these studies may be directly related to the hemolytic mechanism, but the relatively long time required for the gross changes in ATP concentration to occur may weigh against this concept. It is also possible that the altered ATP metabolism occurs coincidentally with other chemical changes which lead directly to hemolysis.

In this study attention has been directed primarily to the changes induced by phenylhydrazine in the concentrations of the adenine nucleotides of the erythrocyte. However, in blood incubated with phenylhydrazine there was also an increase

in the quantity of ultraviolet-absorbing material appearing after ATP in the chromatograms. The chemical nature and biologic significance of this material are at present unknown, although it appears to be at least quantitatively important, since it represented up to 28 per cent of the total ultraviolet-absorbing material in these extracts.

SUMMARY

1. Incubation of normal and glucose-6-phosphate dehydrogenase (G6PD)-deficient blood with phenylhydrazine induced a decrease in adenosine triphosphate (ATP) concentration and a corresponding increase in adenosine diphosphate and adenosine monophosphate. In addition, there was an increase in the amount of ultraviolet-absorbing material of unknown chemical composition which was eluted from Dowex-1 formate resin columns after adenosine triphosphate.

2. Phenylhydrazine enhanced glucose utilization and pyruvate accumulation in both normal and G6PD-deficient blood.

3. The fall in ATP concentration induced by incubating normal blood with phenylhydrazine for 8 hours was largely prevented by adding glucose to the incubation mixture but, in normal blood incubated for 12 hours with phenylhydrazine, a definite fall in ATP concentration was demonstrated even with glucose added.

4. Glucose was less effective in protecting G6PD-deficient blood from the ATP-lowering effect of phenylhydrazine than it was with normal blood.

5. Phenylhydrazine induced a more rapid fall in the ATP concentration of erythrocytes washed free of glucose than occurred in the controls.

ACKNOWLEDGMENT

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PHENYLALANINE METABOLISM AND FOLIC ACID ANTAGONISTS *

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Amethopterin and related folic antagonists inhibit the reduction of folic acid to tetrahydro folic acid (2). The clinical effects of these drugs are thought to be related in turn to the role of tetrahydro folic acid in single-carbon transfer reactions during nucleic acid synthesis (3). Recently, pteridines such as folic acid have been implicated in another area of metabolism, the conversion of phenylalanine to tyrosine. In this conversion the pteridine is thought to act as a cofactor which is reduced in a separate enzymatic reaction, then takes part in the actual hydroxylation of phenylalanine. The reaction sequence as it is currently deduced from studies *in vitro* is presented in Figure 1. Enzymatic activities corresponding to phenylalanine hydroxylase ("rat enzyme") and the cofactor-reducing enzyme ("sheep enzyme"), as well as the cofactor itself, have been observed in normal human liver (4).

It has been shown that folic acid antagonists such as amethopterin inhibit tyrosine synthesis in a partially purified enzyme system, and *in vivo* in the rat (5). The site of inhibition is believed to be the enzymatic reduction of the pteridine cofactor. The work reported here is an investigation of the effects of amethopterin on phenylalanine metabolism in man, and the possible relevance of this effect to the clinical properties of folic antagonists. Experiments were also performed on rodents to explore the possible interrelationship of folic acid metabolism, phenylalanine metabolism, and cancer chemotherapy.

METHODS

Patients receiving amethopterin (Methotrexate) for the treatment of histologically-proven metastatic choriocarcinoma or leukemia were studied before, during and after therapy. Amethopterin was given in either of two dosage schedules: patients with carcinoma received 20 to 25 mg intramuscularly daily for 5 days in repeated courses (6); patients with leukemia received 5 to 10 mg orally daily, as continuous therapy. Several patients were studied who received 6-mercaptopurine orally in daily doses of 100 to 200 mg and several who received vincaleukoblastine, 5 to 10 mg intravenously, each day for 3 days (7). Six adults were studied who did not have known malignant disease and did not receive chemotherapy.

Phenylalanine metabolism was measured by an oral phenylalanine tolerance test. The DL or L form of the amino acid was given after an overnight fast in a dose of 0.1 g per kg body weight mixed in orange-ice. Blood was drawn at 0, 2, 4, and 6 hours after the phenylalanine was given. The serum was analyzed for phenylalanine by the method of La Du and Michael (8), and several determinations confirmed by the method of Kaufman and Levenberg (5). Tyrosine was measured by the method of Udenfriend and Cooper (9).

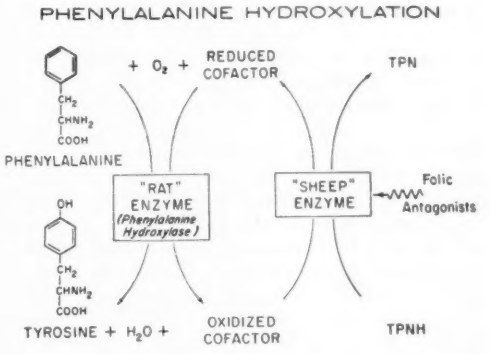

FIG. 1. SCHEMATIC REPRESENTATION OF THE ENZYME SYSTEM FOR HYDROXYLATION OF PHENYLALANINE IN MAMMALIAN LIVER AND THE LOCUS OF INHIBITION BY FOLIC ANTAGONISTS.

Recoveries of added phenylalanine and tyrosine were quantitative from sera of patients before, during and after therapy. The decay constants for serum phenylalanine were calculated for the 2- to 4-hour, and 4- to 6-hour

* A preliminary report of the clinical findings described here has been presented at the Forty-Fourth Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 13, 1960 (1).

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intervals, from the equation

$$k = -\frac{2.303}{2}\log_{10}\frac{10(C_2 - C_4)}{C_2}, \text{ or } \frac{10(C_4 - C_6)}{C_4}$$

where C_2, C_4, and C_6 represent the increment of serum phenylalanine above the fasting level at 2, 4, and 6 hours, respectively. The greater of the two values for k was included in Table I. These calculations assume that serum phenylalanine fell in accordance with a first order rate equation after an oral dose. The assumption is supported by the agreement of the k values for each time interval after the second hour; i.e., the logarithms of the phenylalanine values at 2, 4, and 6 hours fell on a straight line when plotted against time. The other parameters of phenylalanine tolerance were the maximal serum phenylalanine achieved during the test and the increment in plasma tyrosine during the test. Each patient provided her own control before or after therapy. Several tests were performed in which oral L-tyrosine was used in place of phenylalanine, and the data were treated in the same way.

In some studies urine was collected in 6-hour samples on the days of the tolerance tests and assayed for phenylpyruvic acid by the method of Følling and Sydnes (10).

The therapeutic response to the drugs was assessed by hormonal and radiological criteria in the cases of choriocarcinoma, as described by Hertz and co-workers (6), and by hematological criteria in the cases of leukemia. Drug toxicity was assessed clinically. The parameters for toxicity included mucous membrane ulceration, skin eruptions, and bone marrow suppression as reflected in peripheral blood counts.

Several tissues were assayed for the presence of the enzyme, phenylalanine hydroxylase, by using the two independent methods described by Kaufman (11). The tissue samples were homogenized in either 0.01 M acetic acid, or in 0.01 M potassium phosphate buffer, pH 7.0. Simultaneous assays were performed on rat liver as a day-to-day check of the assay procedure.

Folic deficiency in Sprague-Dawley rats was induced by feeding weanling animals either a commercial folic-deficient diet with 2 per cent succinylsulfathiazole (Sulfasuxidine, Nutritional Biochemicals) for 7 weeks, or a high-fat, folic-deficient diet for 16 weeks. The latter diet, developed by Silverman and Pitney, prevents the weight loss usually seen in folic deficiency in rats (12). Controls were given the former diet plus 20 mg of folic acid per kg of diet. Folic deficiency in the first group of animals was documented grossly by failure to gain weight, and by low total white blood cell counts. In the group fed the high-fat diet, deficiency was documented by the elevated urinary excretion of formiminoglutamic acid, as measured by a microbiologic assay. The livers were assayed for folic acid plus precursors with the digestion procedure of Silverman and Gardiner (13), and a microbiological assay employing *Pediococcus cerevisiae*. The same livers were assayed for the cofactor of phenylalanine hydroxylase by the method of Kaufman (14). Assays were performed in the linear range of the cofactor determination.

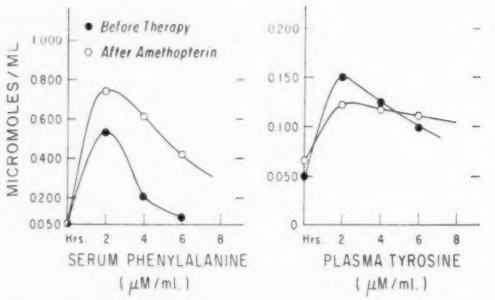

FIG. 2. A REPRESENTATIVE PAIR OF ORAL PHENYLALANINE TOLERANCE TESTS PLOTTED ON LINEAR COORDINATES (PATIENT 1, TABLE I).

A pilot study was performed to test whether changes in the phenylalanine or tyrosine content of the diet affected the response to amethopterin of transplantable mouse leukemia. The diets contained either 1 per cent L-phenylalanine and 1 per cent tyrosine (normal), 3.5 per cent L-phenylalanine and 1 per cent L-tyrosine (high-phenylalanine), or 1 per cent L-phenylalanine and no tyrosine (low tyrosine). The diets were formulated according to Franklin, Stokstad, Belt and Jukes (15), except that the casein hydrolysate was treated to remove aromatic amino acids [1] and supplemented with tryptophan and methionine. Survival times were recorded, and a dose-response curve to amethopterin was determined for each diet after inoculation of the mice with strain L-1210 transplantable leukemia. The general procedure of Goldin, Venditti, Humphreys and Mantel was employed (16).

RESULTS

Phenylalanine tolerance tests. Table I presents the results of the clinical study and includes the toxic and therapeutic effects of amethopterin, and the parameters of phenylalanine tolerance. In some patients repeated courses of therapy and repeated phenylalanine tolerance tests were performed. Every patient demonstrated impaired phenylalanine tolerance after amethopterin. This was manifest by a higher maximal serum phenylalanine level, a slower decay from this maximum (smaller k), and a smaller increment in blood tyrosine. A typical study is presented graphically in Figure 2, and phenylalanine values for several other studies are plotted on semilogarithmic coordinates in Figure 3.

The shortest time interval between the first injection of amethopterin and the performance of a tolerance test was 12 hours, (Patients 5, 6), and

[1] Supplied as compound MJ 309B by Mead, Johnson and Co., Evansville, Ind.

TABLE I

Phenylalanine tolerance tests in human subjects

Patient	Diagnosis	Therapy			Tolerance tests				Clinical effects			
		Drug*	Total drug before test	Interval, last dose and test	Dose and isomer of phenyl.	Max. serum phenyl.†	Decay constant (k) for serum phenyl.	Max. plasma tyrosine†	Fall, gonadotropin excret.	Fall, peripheral WBC count	Toxicity to oral mucosa and skin (0 to +2)	Eventual remission $\bar{p}$ therapy
			mg	*days*	*g*	*μmoles/ml*	*hrs*$^{-1}$	*μmoles/ml*	%	%		
1	Chorio-carc.		0		5.7 L	0.477	0.986	0.101				
		A	125	1.5	5.7 L	0.706	0.642	0.057	0	64	+1	Yes
2	Chorio-adenoma	V	0		5.4 L	0.398	1.070					
			25	0.5	5.4 L	0.438	0.950		59	82	0	
		A	125	3.5	5.4 L	0.891	0.437		32	0	+1	Yes
3	Chorio-carc.		0		4.8 L	0.375	0.891	0.123				
		A	125	0.5	4.8 L	0.675	0.659	0.052	99	46	+1	
		A	375	36.5	4.8 L	0.410	0.976			54	0	Yes
4	Chorio-carc.		0		5.9 DL	0.139	1.060	0.099				
		A	125	0.5	5.9 DL	0.403	0.326	0.050	99	68	+1	
		A	500	102.5	5.9 DL	0.285	0.805	0.082	72	47	0	Yes
5	Chorio-carc.		0‡		5.3 DL	0.245	0.805	0.060				
		A	25	0.5	5.3 DL	0.473	0.614	0.027	99	43	+1	Yes
6	Chorio-carc.		0		6.6 DL	0.324	0.990	0.072				
		A	25	0.5	6.6 DL	0.382	0.104	0.042	99	52	+2	Yes
7	Chorio-carc.		0		4.1 DL	0.073	0.984	0.071				
		A	125	0.5	4.1 DL	0.255	0.904	0.057	99	81	+2	Yes
8	Chorio-carc.		0		6.1 DL	0.152	0.941	0.062				
		A	100	0.5	6.1 DL	0.391	0.283	0.039	0	66	+2	Yes
9	Chorio-carc.		(300)	(120)	4.7 DL	0.453	0.995	0.047				
		A	360	2.5	4.7 DL	0.323	0.758	0.026	86	41	+1	Yes
10	Lympho. leuk.	6-M-P	75/day	0.5	3.4 L	0.398	0.737		0	Marked	0	No
		A	2.5/day × 14	0.5	3.4 L	0.740	0.533		0	0	0	Temporary
11	Chorio-carc.		0		6.4 DL	0.160	0.896	0.055				
		A	240	0.5	6.4 DL	0.413	0.606	0.027	66	0	0	
		A	490	0.5	6.4 DL	0.363	0.622	0.031	30	44	+2	No
12	Chorio-carc.		0		4.4 DL	0.121	0.875	0.068				
		A	250	9.5	4.4 DL	0.453	0.538	0.084	99	33	+2	No
13	Chorio-carc.	A	1,125	1.5	4.9 DL	0.371	0.594		0	0	0	No
		V	20	18.5	4.9 DL	0.207	0.884		72	70	0	No
14	Chorio-carc.	A	550	4.5	4.6 DL	0.277	0.139		0	59	+2	No
		V	63	16.5	4.6 DL	0.192	0.775		0	88	0	No
15	Acute myelo. leuk.		0		8.6 L	0.524	0.894					
		6-M-P	130–250/day × 15	0.5	8.6 L	0.532	0.845			Marked		No
16	Acute lympho. leuk.				6.7 L	0.223	0.995					
		6-M-P	200/day × 12	0.5	6.7 L	0.239	0.746			0		No
17	"Stein-Leventhal"	None			6.2 L	0.258	1.147					

* Drug: A = amethopterin; V = vincaleukoblastine.
† Total serum level minus fasting serum level, except in Patient 25.
‡ 700 Mg amethopterin had been administered over a period of several weeks, 78 days before listed studies were begun.
§ Derived from data published by Hsia and Paine (17).

TABLE I—(*Continued*)

Patient	Diagnosis	Therapy: Drug*	Therapy: Total drug before test	Therapy: Interval, last dose and test	Tolerance tests: Dose and isomer of phenyl.	Tolerance tests: Max. serum phenyl.‡	Tolerance tests: Decay constant (k) for serum phenyl.	Clinical effects: Max. plasma tyrosine†	Clinical effects: Fall, gonadotropin excret.	Clinical effects: Fall, peripheral WBC count	Clinical effects: Toxicity to oral mucosa and skin (0 to +2)	Clinical effects: Eventual remission on therapy
			mg	*days*	*g*	*μmoles/ml*	*hrs*$^{-1}$	*μmoles/ml*	*%*	*%*		
18	Normal	None			6.0 L.	0.200	0.754					
19	Normal	None			6.7 L.	0.512	0.977					
20	Hypogonadism	None			5.3 L.	0.340	0.888					
21	"Stein-Leventhal"	None			7.8 L.	0.695	0.892					
22	Obesity	None			9.0 L.	0.574	1.027					
23§	Normals (mean)	None			0.1 g/kg L.	0.493	0.834					
24§	Heterozygote phenylpyruvics	None			0.1 g/kg L.	0.927	0.535					
25	Phenylpyruvic oligophrenia	None			0.05 g/kg L.	2.633†	9.747–10	0.005				

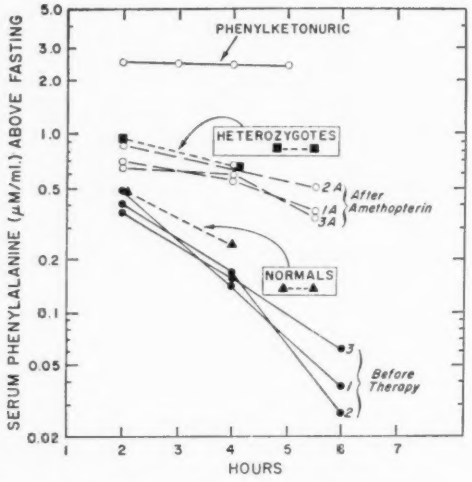

FIG. 3. ORAL PHENYLALANINE TOLERANCE TESTS, PLOTTED ON SEMILOGARITHMIC COORDINATES. The curves labeled 1, 2, 3, and 1A, 2A, 3A are from the corresponding patients in Table I. The suffix "A" indicates tests performed after amethopterin. The curves labeled "heterozygotes" and "normals" are from the data of Hsia and Paine (17). "Heterozygotes" are subjects whose children had phenylpyruvic oligophrenia. The curve labeled "phenylketonuric" is a phenylalanine test performed during the current study on a subject with known phenylpyruvic oligophrenia. All values are corrected for the fasting phenylalanine level except those for the phenylketonuric subject.

a marked change in phenylalanine tolerance was evident at that time. The changes in phenylalanine tolerance were observed 2 weeks after the last injection of amethopterin.

The control tests (before therapy) in patients with cancer did not differ from those in patients without cancer. The fasting levels of phenylalanine and tyrosine did not differ in these groups, nor did they change after therapy. Also included in the table and in Figure 3 are the mean values reported by Hsia and Paine for similar tests in normal subjects and presumed heterozygotes for the gene producing phenylpyruvic oligophrenia (17). One test in the current study was done on a patient known to have phenylpyruvic oligophrenia.

Oral tyrosine tolerance tests were performed in three patients before and after therapy. Amethopterin did not alter the absorption or metabolism of tyrosine as measured by the maximal

plasma level and the rate of decay of plasma tyrosine.

Routine liver function tests (serum bilirubin, alkaline phosphatase, and glutamic-oxalacetic transaminase levels) failed to show consistent changes after amethopterin therapy.

Patients who received 6-mercaptopurine or vincaleukoblastine demonstrated toxic side effects to the drugs which resembled those that followed amethopterin, but none of these patients showed impaired phenylalanine tolerance (Patients 2, 10, 13–16).

Within Table I the patients are arranged roughly in order of decreasing tumor response to therapy. No correlation was found between this response and the degree of impairment of phenylalanine tolerance. Furthermore, no correlation was observed between toxicity from amethopterin and phenylalanine tolerance.

The urine of Patients 1, 3–6, 8, 9, 11 and 12 was tested for phenylpyruvic acid before and after amethopterin. In those patients who received the DL-phenylalanine mixture, there was detectable phenylpyruvic acid in the urine, but the excretion did not rise after therapy. In the patients who received L-phenylalanine, no phenylpyruvic acid was detected before or after therapy.

Assay of phenylalanine hydroxylase in various tissues. Phenylalanine hydroxylase assays were performed on the following numbers of separate tissue specimens: 1 human choriocarcinoma (2 strains) grown as heterologous transplants in the cheek pouch of the hamster (18), human choriocarcinoma excised at surgery; 1 human term placenta; 1 human placenta aborted after 18 weeks' gestation; 3 rat placenta approximately 3 days from term; 4 rat mammary gland during pregnancy; 2 rat mammary gland during lactation; 20 immature rat uterus after 6 days' stimulation by exogenous estrogen; 3 rat small intestine; 1 rabbit marrow; 1 rabbit skin. These tissues were chosen for assay because of their known sensitivity to the effects of folic antagonists. There was no phenylalanine hydroxylase activity detected in any of the specimens tested. Thus, liver is the only tissue in which significant phenylalanine hydroxylase activity has been detected.

Effect of folic acid-deficient diet on cofactor content of liver. Table II presents the findings of an experiment to determine the effect of folic

TABLE II

Effects of folic-deficient diets on the content of cofactor for phenylalanine hydroxylase in rat liver

Rat	On diet	Weight	Peripheral WBC count	FIGLU* excretion	Folic acid content of liver†	Cofactor content of liver‡
	days	*g*		*μmoles/day*	*μg/g wet liver*	*U/g wet liver*
Standard diet + folic acid						
1	51	201	4,700		10.0	0.67
2	51	188			7.5	0.73
3	51	198			9.5	0.89
4	51	211	5,200		8.6	0.81
5	51	188			10.0	0.71
6	51	175	4,300		8.0	0.84
Standard diet, deficient						
7	51	183	2,400		0.48	0.36
8	51	124	3,100		0.47	0.50
9	51	165	1,500		0.50	0.57
10	51	123	2,200		0.30	0.55
11	51	116	2,600		0.49	0.49
High-fat diet, deficient						
12	120	180		133	0.56	0.77
13	120	170		113	0.46	0.80
14	120	180		113	0.49	0.69

* Formiminoglutamic acid; normal urinary excretion for the rat is less than 5 μmoles/day.
† Measured as citrovorum factor after autolysis to convert "prefolic" and folic acids to citrovorum factor (13).
‡ One unit is the amount of cofactor activity leading to the formation of 1 μmole tyrosine in a standard assay containing phenylalanine, phenylalanine hydroxylase, and "sheep enzyme" (14).

acid deficiency on the level of cofactor for phenylalanine hydroxylase in rat liver. There was a 36 per cent reduction in the mean level of cofactor activity in only those rats which demonstrated inanition. The rats fed a high-fat diet to prevent inanition failed to show any reduction of cofactor content.

The pilot study using mouse leukemia failed to yield evidence for an effect of various dietary phenylalanine : tyrosine ratios on prolongation of survival time by amethopterin.

DISCUSSION

The complete phenylalanine hydroxylase system requires two enzymes (named for their convenient animal sources), TPNH, oxygen, phenylalanine, and a cofactor (Figure 1). The exact structure of the cofactor is not known, but it has many of the chemical characteristics of a pteridine. Several synthetic tetrahydro pteridines including tetrahydro folic acid can substitute for the natural cofactor *in vitro*.

The second enzyme ("sheep liver enzyme"), catalyzes the reduction of the cofactor by TPNH (4). In the presence of reduced cofactor and oxygen, phenylalanine is converted to tyrosine in a reaction catalyzed by phenylalanine hydroxylase ("rat liver enzyme"). Folic antagonists are thought to block the enzymatic reduction of cofactor in a manner analogous to their inhibition of the activation of folic acid coenzymes by folic acid reductase (2). The experiments described here suggest that the scheme of phenylalanine hydroxylation derived from *in vitro* investigation applies as well to the human.

The inhibition of phenylalanine metabolism by amethopterin therapy is incomplete. There is, possibly, nonenzymatic reduction of the cofactor, unaffected by amethopterin, which permits phenylalanine metabolism to proceed at a reduced rate after inhibition of sheep liver enzyme. The observed inhibition of phenylalanine metabolism is of the same order of magnitude as the impaired phenylalanine metabolism found by Hsia and Paine in heterozygotes for phenylpyruvic oligophrenia (17) but far less than that seen in the homozygote. It should be emphasized that whereas amethopterin blocks sheep liver enzyme, patients with phenylpyruvic oligophrenia lack the hydroxylating enzyme itself (rat liver enzyme). The "sheep" enzyme and cofactor are present in normal amounts in the liver of patients with this disease (4).

There is little resemblance in the toxic state following folic antagonists to the clinical picture of phenylpyruvic oligophrenia. No evidence was obtained for characteristic mental or dermatologic changes. In addition, the patients with drug-induced impairment of phenylalanine metabolism did not excrete phenylpyruvic acid in the urine. This is consistent with the finding of Armstrong and Low, who failed to detect phenylpyruvic acid in the urine unless the serum phenylalanine level exceeded 0.91 μmoles per ml (19).

The results of our study, with regard to the speed of onset and duration of changes in phenylalanine tolerance after amethopterin, are in accordance with those found by Condit for folic reductase activity (20). He measured the excretion of citrovorum factor after a test dose of folic acid and found an effect within 3 days of an injection of amethopterin. The effect was evident for 2 to 10 weeks after the last dose of amethopterin, depending on the dosage schedule. Charache, Condit and Humphreys (21) found persistence of amethopterin, detected by direct assay of human liver, 3 months after the last dose of amethopterin.

Three experiments were performed to assess the importance of the "phenylalanine effect" of amethopterin in chemotherapy, and all three were negative. First, there was no correlation between the degree of impaired phenylalanine tolerance and the clinical response to treatment. Second, there was no effect from feeding additional phenylalanine or omitting dietary tyrosine on amethopterin therapy in mouse leukemia. These dietary alterations were designed to accentuate the probable metabolic consequences of inhibition of phenylalanine hydroxylation. Third, there was no evidence for the presence of phenylalanine hydroxylase in tumor tissue or in normal tissues (other than liver) which are known to be affected by folic antagonists.

The folic-deficient diet failed to reduce the hepatic level of cofactor for phenylalanine hydroxylase except when inanition was also induced. The folic acid level was consistently reduced. This

may indicate that the cofactor is not derived from folic acid, or that the cofactor is preferentially retained in the liver under conditions of folic deficiency, or both.

Hydroxylation is the first step in the sequence of reactions that convert phenylalanine to CO_2 via tyrosine and homogentisic acid. The reaction may be considered, therefore, part of a degradative pathway for excessive phenylalanine, as well as a part of several synthetic pathways which include tyrosine. Folic antagonists also inhibit the degradation of the amino acid histidine by blocking the metabolism of formiminoglutamic acid (FIGLU) (22). The excretion of this intermediate has been used as a test of folic deficiency or folic antagonism. There is no evidence that FIGLU excretion correlates with the therapeutic effects of amethopterin. Thus, three biochemical tests are available for the assessment of folic antagonism in mammals: the failure to excrete normal amounts of citrovorum factor after a test dose of folic acid, the excretion of abnormally large amounts of formiminoglutamic acid after the feeding of histidine, and the impairment of phenylalanine metabolism during an oral tolerance test.

SUMMARY

1. Amethopterin therapy interferes with phenylalanine metabolism in man, as measured by the oral phenylalanine tolerance test.

2. No evidence could be obtained for a relationship between impaired phenylalanine metabolism and the therapeutic or toxic effects of amethopterin in man or in the mouse.

3. Pronounced folic deficiency in the rat is sometimes accompanied by a slight reduction in the level of the cofactor for phenylalanine hydroxylase.

4. These findings have been discussed with reference to the proposed scheme of phenylalanine metabolism and the enzymatic effects of amethopterin in man.

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PHENYLTHIOCARBAMIDE NON-TASTING AMONG CONGENITAL ATHYROTIC CRETINS: FURTHER STUDIES IN AN ATTEMPT TO EXPLAIN THE INCREASED INCIDENCE *

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Phenylthiocarbamide (PTC) is a chemical compound which is bitter-tasting to approximately 70 per cent of white North Americans (1). The remaining 30 per cent has a discrete reduction in taste threshold. This non-tasting trait is inherited as a probable mendelian autosomal recessive (2–4). A common thionamide group structurally relates phenylthiocarbamide to thiourea, propylthiouracil and goitrin (see Figure 1). These four substances are also similar in their ability to interfere with the synthesis of thyroid hormone, an action which leads to thyroid hypertrophy.

PTC non-tasting has been shown to be more common in adult euthyroid patients with goiters (5, 6). In a preliminary report (7), we have presented evidence that the incidence of non-tasting among congenital athyrotic cretins is significantly increased. The purposes of the present communication are to relate the taste threshold of these cretins to their intellectual development, birth weight, birth month, and race, to describe more fully the methods and clinical findings, and to demonstrate that the distribution by taste thresholds for goitrin, a naturally occurring goitrogen, is simliar to phenylthiocarbamide.

METHODS

The subjects and controls were tested by the author with 14 concentrations of phenylthiocarbamide, and the method used was that of Harris and Kalmus (8). Dilution 1 contains 1.3 mg of PTC per ml; each subsequently numbered solution is one-half the strength of the preceding concentration. If a subject is able to taste only one of the concentrated solutions (1 through 4), he is designated a non-taster, whereas if his threshold is at dilution 5 through 14, he is a taster. Plastic squirt bottles facilitated the procedure. By testing the parents first, the interest and cooperation of the child was often stimulated. For the adults, the taste threshold was determined by finding the weakest concentration at which they could differentiate 4 beakers of water from 4 beakers of the test concentration. In children and infants, the concentration of solutions was increased until bitterness was detected by vocal response and grimace. This was repeated in most instances until the threshold was established by 3 responses at the same concentration. In Figure 2 the reproducibility of the test in children is illustrated. The average age in this group was 7.0 years.

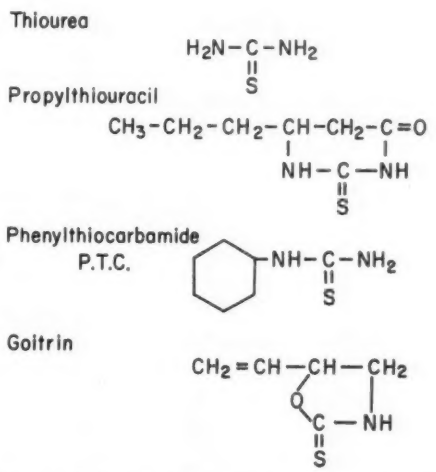

FIG. 1. CHEMICAL FORMULAS OF THE PERTINENT THIONAMIDES.

Thresholds for taste of propylthiouracil, thiourea and goitrin [1] were determined in adult volunteers by eliciting

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[1] The goitrin was in the *d,l* form and was a gift from Dr. M. R. Altamura of the Pioneering Research Division, Quartermaster Research and Engineering Center, United States Army, Natick, Mass.

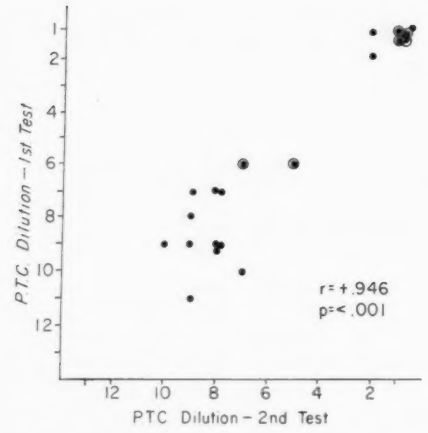

FIG. 2. REPRODUCIBILITY OF PTC THRESHOLDS IN CHILDREN. The circled points indicate retested athyrotic cretins.

a response on 3 serial passages through the endpoint. The concentrations used are detailed in Figures 5, 6, and 7.

An analysis of the maternal dietary intake of antithyroid foods was undertaken by the use of questionnaires. The number of monthly servings for 20 substances was estimated by the mother for the usual diet and compared with the diet during pregnancy. Ten of these 20 foods are known to contain goitrin or to be thyroid-inhibiting in man (9-14) (Table I). Responses were obtained from 27 of the 30 mothers of cretins, and they were compared with 17 similar questionnaires completed by mothers of mentally retarded children.

TABLE I

Foods in maternal diets investigated by questionnaire

Food	Antithyroid effect (10, 11,13)	Goitrin isolated (9, 11, 12, 14)
Rutabaga	+	+
Turnip	+	+
Cabbage	±	+
Brussel sprouts	−	
Broccoli	+	
Peaches	+	
Pears	+	
Strawberries	+	
Spinach	+	
Peanuts	±	
Carrots	+	
Corn	−	
Cauliflower	−	
Pickles		
Peas	±	
String beans	±	
Soybeans	+	
Milk	±*	+*
Lettuce	±	
Mustard	−	

* When goitrin-containing substances were administered to the cow.

TABLE II

Summary of clinical findings

Case	Sex	Birth date	Age tested		Birth wt	Typical history	Treatment before 6 mo	IQ	PBI	24-Hr I^{131}	PTC threshold			
											Patient	Mother	Father	Siblings
		mo/yr	*yrs*	*mos*	*g*				*μg/100 ml*	*%*				
1	F	9/52	6	10	2,380	+	−	50		3	?	5	8	11,9
2	F	8/58	1		2,380	+	+	109	3.2	0.3	6	7		
3	M	7/55	4		2,870	+	+	78	3.2	3.3	1	3	10	
4	F	6/55	4	1	3,150	+	−	64	2.7	0	?	11	9	8
5	F	5/55	4	2	3,600	+	+	normal*			1	1	3	
6	F	10/57	1	9	5,100	+	+	52	3.5	1.6	1	1	1	
7	F	1/54	5	6	3,820	+	+	85		0	6	6		7
8	M	1/56	3	6	1,920	+	−	38	2.8	6.1	?	4	10	3
9	F	9/55	3	10	3,490	+	+	100	3.1		1	2	8	
10	F	9/58		11	3,150	+	+	normal*	2.3		1	1	7	
11	F	9/46	12		3,610	+	+	74			1	1	6	1,2
12	F	6/57	2	2	4,080	+	+	50–60	1.0	5	6	8	9	7
13	F	2/47	12	6	3,080	+	+	117			1	1		3,2,4,1,1,8
14	F	10/57	1	10	4,600	+	+	124			8	11	5	8
15	M	5/56	3	3	3,600	+	+	95	1.6		6	10	7	10
16	M	5/56	3	3	4,200	+	+	113	2.2		7	10	7	10,4
17	M	1/58	1	8	3,100	+	+	low normal*	1.0		2	1	7	
18	F	9/55	4		3,610	+	+	82	1.2	1	1	4	4	1
19	M	1/56	3	8	2,950	+	+	60	1.0	10	1	7	1	
20	F	11/53	5	10	3,610	+	−	38	1.0		?	8	1	
21	M	1/58	1	8	3,940	+	+	low normal*	0.6	8	6	9	10	
22	F	5/55	4	5	3,200	+	−	106	1.0	8	1	1	1	1,1
23	F	1/47	12	8	2,950	+	+	101			2	1	1	2
24	F	3/53	6	6	3,820	+	+	85	0.8	low	8	9	7	
25	M	2/56	3	6	4,170	+	+	92	2.0	0	10			
26	F	8/47	12	1	3,220	+	−	60		low	1	1	1	1
27	M	12/51	7	9	4,000	+	+	105			1	8	1	2,4
28	F	12/50	8	9	3,440	+	+	48	3.4		1	1	2	5
29	F	8/49	10	1	3,900	+	−	83		low	1	9	1	12
30	M	9/44	15		4,000	+	−	60		low	1	7	8	1
31	M	6/47	12	3	3,060	+	−	85		low	1	1	1	

* Not tested by formal psychometric examination.

The psychometric evaluations of intelligence were performed by different examiners in 27 of the patients. The remaining 4 children were not formally tested.

Correlation coefficients and χ^2 tests were calculated with the formulas of Edwards (15).

Clinical material. The 31 congenital athyrotic cretins and their families were from the clinics of university hospitals in several sections of North America.[2] The diagnosis was based on typical signs and symptoms of cretinism which appeared during the first 4 months of life. All of the patients had a history of enlarged tongue, myxedema, umbilical hernia, growth arrest, and dramatic response to thyroid therapy. If the history suggested an acquired type of cretinism (i.e., cutting teeth or normal growth and development during the first year), the patient was classified before taste testing as having acquired hypothyroidism. In 25 of the 31 congenital athyrotic cretins studied, either a serum protein-bound iodine or an I^{131}-thyroidal accumulation, or both, supported the diagnosis (Table II). There were 20 females and 11 males; Subjects 24 and 25 were brother and sister. Only one child was living in a school for the mentally handicapped.

The control group consisted of adults working in the University of Washington Medical School and children attending out-patient clinics of the University Hospital, and the Children's Orthopedic Hospital of Seattle. None of the controls had thyroid disease.

RESULTS

Athyrotic cretins. The results of PTC testing are shown in Table II and Figure 3. In four of the cretins, the threshold could not be accurately determined. Of the remaining 27, 18 were non-tasters and 9 were tasters; in the control group, which consisted of 42 adults and 62 children, there were 29 non-tasters (Figure 3). The incidence of non-tasting in the cretinous group was significantly elevated [$\chi^2 = 22.0$, degrees of freedom (D.F.) = 1, $p < 0.001$].

In a group of 13 children with acquired hypothyroidism, 4 were PTC non-tasters. Three of the 13 children had a goitrous form of cretinism and were tasters.

The intelligence quotients of the tasters are compared with the non-tasters in Figure 4. There

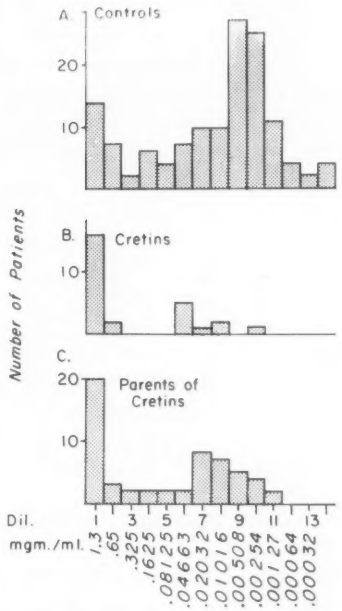

FIG. 3. THRESHOLD DILUTIONS OF PTC FOR THE MEMBERS OF THREE GROUPS. Dilutions 1 to 4 designate a non-taster; dilutions 5 to 14 designate a taster.

was no statistically significant correlation between the IQ rating and PTC-tasting threshold, but 7 of the 15 non-tasters had a ratio below 80 whereas only one of the 8 tasters was below 80. When the 9 patients who were treated after 6 months of age were excluded from the sample, there was no increase in significance of correlation.

No relationship between taste status and the year or month of birth was noted.

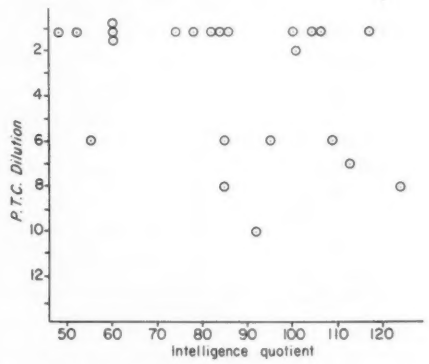

FIG. 4. INTELLIGENCE QUOTIENTS OF THE FORMALLY TESTED CRETINS ARE COMPARED WITH THEIR PTC THRESHOLD.

[2] Subjects 1–11 were from the Pediatric Endocrine Clinics of the University Hospital and of the Children's Orthopedic Hospital, Seattle, Wash.; 12–16 were made available by Dr. Geoffrey Robinson, Dept. of Pediatrics, Univ. of British Columbia, Vancouver, B. C.; 17–21 were from the clinic of Dr. John Crigler, Dept. of Pediatrics, Harvard Medical School, Boston, Mass.; 22–31 were from the clinic of Dr. Lawson Wilkins, Dept. of Pediatrics, Johns Hopkins Hospital, Baltimore, Md.

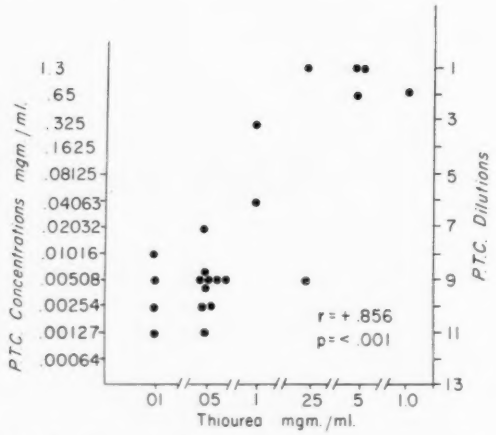

FIG. 5. TASTE THRESHOLDS FOR PTC AND THIOUREA.

Although the average birth weight in the non-taster group was 3,465 g as compared with that of the tasters (3,845 g), the difference was not significant.

Families of the cretins. The incidence of PTC non-tasting among the parents was significantly elevated ($\chi^2 = 21.6$, D.F. = 1, $p < 0.001$). Of 30 mothers who were tested, 15 were non-tasters and 12 of the 27 tested fathers were non-tasters; 18 of the 29 unaffected siblings were non-tasters.

All of the parents were Caucasian but one, who was Negro. One parent was of Mediterranean ancestry; the remainder were descended from persons who had migrated from northern European countries.

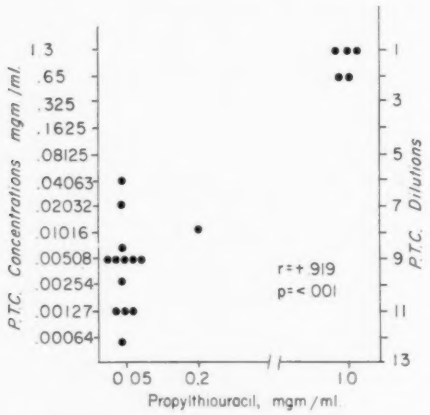

FIG. 6. COMPARISON OF TASTE THRESHOLDS FOR PTC AND PROPYLTHIOURACIL.

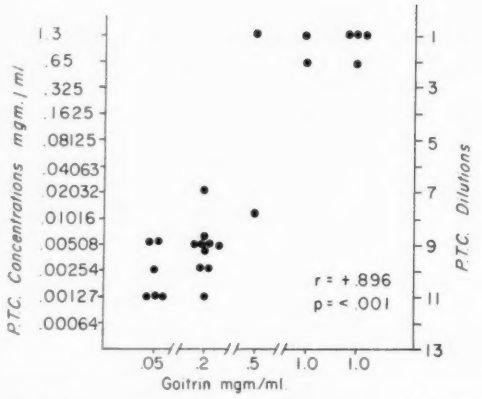

FIG. 7. COMPARISON OF TASTE THRESHOLDS FOR PTC AND *d,l* GOITRIN.

Of the total number of 99 pregnancies reported by the mothers, 12 (12.1 per cent) ended in abortion.

Thyroid disease in the parents was assessed by history and, where possible, augmented by examination of the subject's neck; in four, clear evidence of thyroid hypertrophy was evident. One of these parents had been thyroidectomized for hyperthyroidism; the others were euthyroid.

An analysis of the questionnaires covering maternal dietary intake of antithyroid foods revealed that the mothers of cretins showed no differences from the control group of mothers of mentally retarded noncretinous children.

Taste thresholds for other thionamides. The taste thresholds for thiourea, propylthiouracil and goitrin were compared with the PTC thresholds in the same volunteers. In each case, a significant positive correlation was obtained. The correlation coefficients and concentrations used are detailed in Figures 5, 6 and 7.

DISCUSSION

Although attempts to define PTC non-tasting biochemically or physiologically (16, 17) have been made, no definition has been firmly established. The perception of PTC is localized to the group of taste buds on the posterior aspect of the tongue. Harris and Kalmus (18) have previously shown that other thionamides such as thiourea and propylthiouracil exhibit the same taste difference as PTC. Our findings support their observations and extend this group of thionamides

to include goitrin (*l*-5-vinyl-2-thio-oxazolidone) which is a naturally occurring antithyroid substance (9–14).

A significantly increased incidence of PTC non-tasting has been demonstrated in this group of athyrotic cretins. Because the non-tasting trait is a probable mendelian recessive (2–4), the finding of an increased incidence of non-tasting among the parents and among the siblings helps to exclude the possibility that the testing is inaccurate due to the lowered mentality among cretins. Fraser (19) has tested with PTC 17 of 28 children with a presumptive diagnosis of athyrotic cretinism; 15 were non-tasters.

If cretinism is more common among non-tasters, one would expect fewer cases of congenital hypothyroidism in populations with a lowered incidence of the trait. Non-tasting occurs in only 2 to 10 per cent of the Negro race as compared with an incidence of 30 per cent in non-colored North Americans (20). Publications by Scott and Jenkins (21) and by Childs and Gardner (22) have emphasized that cretinism is uncommon in the Negro race. Scott (23) reports that only two cretinous patients have been admitted to the Pediatric Service of Freedman's Hospital during a 20-year period. A high proportion of patients in this hospital is colored. Childs and Gardner (22) observed only two Negroes in a group of 90 cretins from the Johns Hopkins Hospital. The author has been unsuccessful in learning the incidence of cretinism in other racial populations which have a high percentage of non-tasters, and would appreciate receiving information on this point.

A possible relationship between PTC non-tasting and other features of cretinism was noted only in the case of intelligence quotients, but here the increased intelligence of the tasters over the non-tasters is only suggestive. It was found that the five patients examined in British Columbia (Cases 12–16) were more intelligent and had normal incidence of PTC tasting. A separate pediatrician referred each of these patients, and, therefore, their selection differed from that in the other groups where the sample included a large majority of the cretins attending a particular clinic. It might be natural for an individual pediatrician to refer his most successfully treated patient and thereby possibly select out a different group. On the other hand, the histories of these patients did not differ from the other cretins. It may be pertinent at this point to mention that children with a later developing form of cretinism (goitrous or non-goitrous) had a normal incidence of non-tasting and generally normal mental development.

Hypotheses based on findings. What is the connection between the PTC non-tasting genotype and athyrotic cretinism? Because of the chemical similarity between PTC and other antithyroid drugs and because of the increased susceptibility of non-tasting adults to thyroid disease (5, 6), it is tempting to speculate that the fetus with non-tasting genotype is more susceptible to thyroid maldevelopment. Since the non-tasting state occurs in the normal siblings of the cretins, the PTC non-tasting by itself does not seem to account for athyrotic cretinism but may lead to circumstances which affect organogenesis of the thyroid. There is no evidence from our questioning that the mothers of the cretins have a higher intake of goitrin-containing foods. It should be pointed out that, although some vegetables contain significant amounts of goitrin (9–13), Greer and Deeney have shown that it exists as its glycoside, progoitrin (24, 25). Progoitrin which elicits antithyroid activity (25) is only slightly better and as a result might be unknowingly ingested in large amounts.

A mechanism that might lead to thyroid maldevelopment is an inability of the PTC non-taster to normally detoxify thionamide goitrins such as goitrin. Maloof and Spector (26) have shown that the particulate fraction of sheep thyroid cells has a desulfurating mechanism for the thionamide, thiourea. Similar preparations from sheep liver and kidney did not desulfurate. We have shown that a particulate fraction from human thyroid desulfurates S^{35}-thiourea (27). If the PTC non-taster were unable to desulfurate thionamides, his thyroid metbolism might be differentially affected by these substances.

Although thionamides can cross the mammalian placenta and exert an inhibiting effect on fetal thyroid, there is no experimental evidence in animals (28–37) or in man (37, 38) that they can prevent thyroid embryogenesis.

SUMMARY

The incidence of non-tasting for phenylthiocarbamide (PTC), a mendelian recessive trait, is significantly increased among congenital athyrotic cretins and their families. Speculations to explain this finding are discussed. There is no evidence that the maternal intake of goitrogenic foods was abnormal.

An insensitivity to taste perception for goitrin, thiourea, and propylthiouracil was demonstrated in volunteers who were also non-tasters for PTC.

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PHENYLALANINE METABOLISM IN THE PHENYLPYRUVIC CONDITION. I. DISTRIBUTION, POOL SIZE, AND TURNOVER RATE IN HUMAN PHENYLKETONURIA *

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The inborn metabolic error in phenylpyruvic oligophrenia consists of the inability to hydroxylate phenylalanine to tryrosine in any significant amount. Recent work has focused mainly on the investigation of this hydroxylating system, its purification and mode of action (1–5), the inhibitory effect of phenylalanine and its derivatives on enzymes (6–9), and the prevention of mental retardation by a diet low in phenylalanine (10, 11).

With this diet the free phenylalanine of plasma and total body fluid can be adjusted to any value that might be required to study the effect of phenylalanine concentration on metabolic processes *in vivo*. Bickis, Kennedy and Quastel (12), for example, demonstrated that phenylalanine, at concentrations similar to those observed in the blood of phenylketonuric patients, inhibited the enzymatic degradation of tyrosine *in vitro*. We, on the other hand (13), were unable to find inhibition of the breakdown of orally administered *p*-hydroxyphenylpyruvic acid in patients with plasma concentrations of phenylalanine as high as 50 mg per 100 ml. This discrepancy could be explained, for instance, by a diminished rate of *p*-hydroxyphenylpyruvic acid formation from tyrosine in the *in vitro* experiments, or by a lower intracellular phenylalanine concentration in our *in vivo* study than one would have expected from the plasma phenylalanine levels. Since the distribution coefficient of phenylalanine between tissue and plasma has not yet been determined *in vivo*, the intracellular phenylalanine concentrations in our experiments were not known. In order to evaluate properly the significance of metabolic experiments with phenylalanine, some estimate must be made of the distribution of the phenylalanine between plasma and tissue.

Since patients with phenylpyruvic oligophrenia demonstrate a defined metabolic variation from the norm, they also provide the opportunity to obtain further information that is not readily available in normal subjects. For example, pool size determinations of amino acids have rarely been performed and have mostly proved to be unsatisfactory, because they are based on experiments with N^{15}-labeled amino acids and many assumptions have to be made [see Wu, Sendroy and Bishop (14, 15) and Tschudy and co-workers (16)]. Experiments similar to ours with S^{35}-labeled methionine in nonphenylketonuric individuals have been published by Maurer (17). To our knowledge no studies on human beings have been made, so far, with C^{14}-labeled amino acids, except by Gutman and co-workers (18), who estimated the pool size of glycine. The method used by them, however, was entirely different from ours.

The use of C^{14}-labeled amino acids eliminates the necessity of assuming, as did Sprinson, Rittenberg and San Pietro (19, 20), that the rate of nitrogen transfer is faster than other metabolic processes involved here. It will be shown later that we have to make a similar assumption; namely, that equilibration of the labeled phenylalanine within the body is faster than other processes. If it be assumed that the conversion of phenylalanine to tyrosine is negligible in the penylketonuric patient, then phenylalanine can only be stored, incorporated into proteins, excreted as such or as its metabolites—phenylpyruvic acid and phenylacetic acid.

That this assumption is correct will be shown in a second paper (21). In addition, no appreciable amount of phenylpyruvic acid or phenylacetic acid has been found in the circulating plasma of phenylpyruvic patients in comparison with the total amount of free phenylalanine (22). Therefore, the pool size, the turnover time, and the absolute turnover rate of free phenylalanine pool

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can be estimated by means of C^{14}-labeled phenylalanine without isolating free phenylalanine from the plasma. The absolute turnover rate in the phenylketonuric organism is, therefore, only the sum of incorporation into proteins, excretion, and conversion to compounds other than tyrosine. However, a small, nonspecific conversion to tyrosine might be included. In respect to many metabolic functions, phenylketonuric patients seem to behave like normal individuals; but from a metabolic point of view they may be considered human mutants. Thus, it might be possible to carry out studies at normal phenylalanine levels, taking advantage of the existence of the metabolic block, to derive data applicable to persons other than phenylketonurics.

MATERIALS AND METHODS

Two male and two female patients suffering from phenylpyruvic oligophrenia were studied. Three (J.I., N.C. and K.H.) had been on a diet low in phenylalanine for at least 3 weeks prior to the experiments. High protein foods were replaced by a casein hydrolysate from which phenylalanine had been removed (Lofenalac, Mead Johnson Co.). The plasma phenylalanine was adjusted to levels of 2.3 mg in J.I., 7.0 mg in N.C., and 10.5 and 17.0 mg per 100 ml in $K.H._1$ and $K.H._2$ (the experiments in K.H. were carried out on two different occasions). No phenylpyruvic acid was excreted in the urine as checked by the ferric chloride test. Patient R.S. was maintained on a normal diet, excreted large amounts of phenylpyruvic acid, and had a plasma phenylalanine concentration of 47 mg per 100 ml plasma. The experiments were carried out on 3 consecutive days, in the morning. Breakfast was omitted.

First day. Determination of antipyrine space was done according to Brodie, Axelrod, Soberman and Levy (23).

Second day. A needle with a three-way stopcock was inserted into the cubital vein and a blood sample was taken for the determination of the free phenylalanine plasma level, total plasma proteins, hematocrit, and hemoglobin. About 8×10^6 cpm to 11×10^6 cpm of uniformly labeled L-phenylalanine-C^{14} [1] (specific activity 3.2×10^7 cpm per mg), dissolved in 30 ml of physiological saline, was injected through the needle and the syringe was washed three times with about 10 ml of saline from an attached infusion bottle. A constant saline infusion was started for 5 to 6 hours (the duration of the experiments), the total amounting to not more than 350 ml.

Heparinized blood samples were taken at short intervals for 5.5 hours; in Patient $K.H._1$ for 48 hours. The heparinized blood samples were centrifuged at 700 RCF for 10 minutes. In order to determine the radioactivity of the free L-phenylalanine in deproteinized plasma, 1.0 ml of plasma was added to 2.0 ml of 10 g per 100 ml of an aqueous solution of trichloroacetic acid (TCA).

The resulting precipitate was centrifuged at 1,400 RCF for 20 minutes and a duplicate 1.0 ml of the supernatant was plated on plastic planchets, 7.1 cm^2 in area, and dried at room temperature for counting. The samples were counted in a gas-flow end-window counter with a Micromil window and an efficiency of about 22 per cent. All values were corrected for background and self-absorption and given in counts per minute for 1.0 ml of plasma.

The latter values were plotted on semilogarithmic paper against time, and the resulting first straight line was extrapolated to zero time (t_0). This zero time value was used to calculate the distribution volume by the formula:

$$\frac{\text{cpm injected}}{\text{cpm per 1.0 ml protein-free plasma at } t_0} \qquad [1]$$

The pool size of the free phenylalanine is the product of the distribution volume and the free phenylalanine plasma concentration in milliliters.

The slope of the first straight line, so obtained, permitted the estimation of the half-life time and the turnover time of the pool. The turnover time, as used here, is the reciprocal of the slope constant. The absolute turnover rate is given by the quotient of milligrams of free phenylalanine pool to turnover time in minutes. The average concentration in 1.0 ml extravascular antipyrine space was derived from the following equation by Dost (24):

$$a_{\text{tissue}} = \frac{\text{amount injected} - a_{\text{plasma}} \times V_{\text{plasma}}}{V_{\text{antip.}} - V_{\text{plasma}}} \qquad [2]$$

where a_{tissue} = average apparent phenylalanine concentration in cpm $\times$ ml^{-1} extravascular antipyrine space at t_0; a_{plasma} = apparent phenylalanine level in cpm $\times$ ml^{-1} plasma at t_0; V_{plasma} = Evans blue space in ml; $V_{\text{antip.}}$ = antipyrine space in ml; and the distribution quotient is given by:

$$\frac{a_{\text{tissue}}}{a_{\text{plasma}}} \qquad [3]$$

Third day. Plasma volume determinations were carried out by injecting 5.0 ml of 0.5 per cent aqueous solution of Evans blue (Warner-Chilcott).

Blood samples were taken at 10, 20, and 30 minutes, as well as before injecting the dye. The heparinized blood was centrifuged at 700 RCF and the clear, nonhemolyzed supernatant was measured at 600 mμ in the Beckman DU spectrophotometer. The resulting optical densities were plotted on linear graph paper and extrapolated to zero time. From the value so obtained the dilution of the dye was calculated.

In Patient N.C. the plasma volume was estimated to be 1/22 of the body weight. Determination of free phenylalanine was carried out by means of an enzymatic method by La Du and Michael (25); total plasma protein was determined with the biuret technique (26).

[1] Nuclear Chicago. This corporation claims that their L-phenylalanine-C^{14} has a radiochemical purity of 100 per cent, and a chromotographic and electrophoretic purity of about 99 per cent.

RESULTS AND DISCUSSION

The first part of the disappearance curve reflects the equilibration of the labeled phenylalanine among blood, extracellular space, and intracellular space of a multitude of compartments, as well as the turnover of the labeled phenylalanine. The term "turnover" as used here refers to all metabolic processes, including incorporation into proteins and excretion, but excluding the initial equilibration. A first equilibrium of the isotope is reached in J.I., N.C., K.H.$_1$, and K.H.$_2$ between 20 and 30 minutes, and in Patient R.S., with the high phenylalanine level of 47 mg per 100 ml plasma, at about 90 minutes (Figure 1); from there a straight line was obtained for some time during the course of the experiment. If we assume that at the beginning of this first straight line (the second part of the disappearance curve) the equilibration processes have essentially come to an end, then the turnover of the labeled amino acid can be estimated solely from the first straight line.[2] This assumption finds some support in comparing the antipyrine space and the phenylalanine space as derived from the extrapolation of the first straight line to zero time; both spaces are of the same order of magnitude. The quotient of phenylalanine space to antipyrine space varied from 0.80 to 1.53 with an average of 1.04. This indicates that probably no essential enrichment of phenylalanine, if any, above plasma phenylalanine levels has taken place. However, it should be emphasized that the values for the phenylalanine space and the phenylalanine pool are only approximations. This applies especially to Patients J.I. and N.C. in whom the relatively rapid turnover and the large space of distribution, which requires significant time for equilibration of the radioactive phenylalanine (about 20 minutes), mean that the processes of mixing and turnover may proceed simultaneously at comparable rates. Since turnover probably occurs only, or primarily, within cells, there is little turnover of labeled phenylalanine going on during the very first part of the

[2] The term "first straight line" does not necessarily imply that a second straight line is following. It means only that this is the first part of the curve that appears as a straight line, and the rate of the corresponding process can be described as a first-order reaction. Possibly, the rate given by the first straight line represents the sum of several turnover rates.

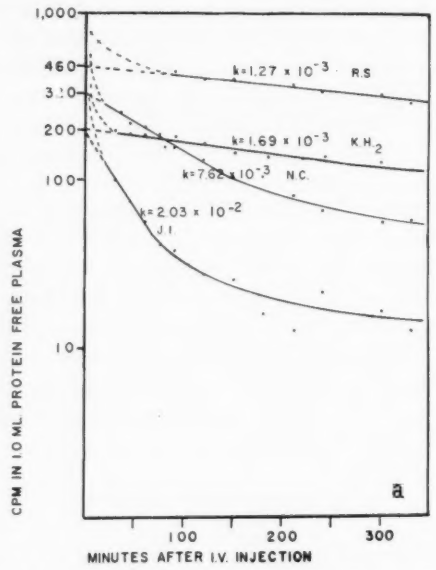
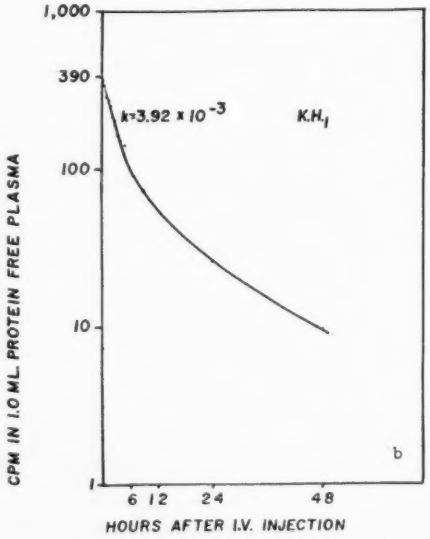


FIG. 1. a) TIME COURSE OF THE DISAPPEARANCE OF FREE L-PHENYLALANINE-C^{14} FROM DEPROTEINIZED PLASMA IN PATIENTS J.I., N.C., K.H.$_2$, AND R.S. All values are corrected for a single interavenous injection of 7.6×10^6 cpm. The experimental values of the dotted curved lines are not shown in the diagram. The straight lines were drawn by inspection of the points. Only in less clear situations was the method of least squares applied; k = first-order reaction rate constant of the first straight line in minutes^{-1}.



b) AS ABOVE, BUT IN PATIENT K.H.$_1$ 1.115×10^7 cpm was injected; k is again given in minutes^{-1}.

plasma disappearance curve. Therefore, the extrapolation of the first straight line, which carries with it the implication that turnover is constant throughout the earlier part of the curve, must introduce some error in the phenylalanine space. This criticism is true of all slope-intercept methods, but is serious only when turnover is rapid compared with the time required for mixing in the pool. We have calculated that the error does not exceed about 15 per cent for J.I. and 6 per cent for N.C. The estimation of the free phenylalanine pool in the intravascular plasma compartment is easy if the plasma volume is determined as Evans blue space. From there the flux in or out of the plasma compartment by turnover may be calculated from the equation:

$$\text{flux}_{\text{plasma}} = V_{\text{plasma}} \times c_{\text{plasma}} \times k \qquad [4]$$

where c_{plasma} is the concentration of free phenylalanine in milligrams per milliliter plasma and k the slope constant of the first straight line ($k = 1/t_t = 0.693/t_{\frac{1}{2}}$). All values are considered to be in fairly good agreement for this kind of experiment from one patient to the other—that is, ± 30 per cent from the average and given in Table I as milligrams free phenylalanine leaving the intravascular compartment per minute or returning to it. Strictly speaking, influx and efflux of the intravascular compartment are not equal, since the system under consideration is leaking; that is, some free phenylalanine is lost with the urine. However, for the first 6 hours the radioactivity excreted amounts to only 2 to 5 per cent of the total dose injected. A substantial part of it might have been excreted during the early phase of equilibration at a time when the specific activity of the free phenylalanine in plasma was relatively high. Since we did not want to catheterize our patients, no early urine specimens were obtained for verification of this assumption.

Maurer (17) likewise found no evidence for significant intracellular concentration of S^{35}-methionine in humans or rabbits, but the situation with respect to other amino acids might be quite different (27). Noall, and Christensen and their co-workers have shown that the nonmetabolizable amino acid, α-amino*iso*butyric acid is many-fold concentrated in human muscle (28) and in various rat tissues (29). In experiments similar to those described above in which α-amino*iso*butyric acid

TABLE I

Summary of values obtained in the patients

Patient	J.I. ♀	N.C. ♀	K.H.₁ ♂	K.H.₂ ♂	R.S. ♂
Age (*yrs*)	23	24	17	17	16
Weight (*kg*)	63.4	51.0	60.0	53.5	36.0
Height (*cm*)	152	149	178	178	144
Plasma proteins (*g/100 ml*)	6.8	7.5	7.2	6.6	6.9
Hemoglobin (*g/100 ml blood*)	14.3	12.0	11.8	14.3	13.5
Hematocrit (%)	43	44	39	46	40
Antipyrine space ($ml \times 10^4$)	2.8	2.7	3.5	3.1	2.1
Evans blue space ($ml \times 10^3$)	2.4	2.3	3.1	2.8	1.4
Free plasma phenylalanine (*mg/100 ml*)	2.3	7.0	10.5	17.0	47.0
Phenylalanine injected ($cpm \times 10^6$)	8.3	7.6	11.2	7.6	7.8
Phenylalanine space ($ml \times 10^4$)	4.1	2.4	2.9	3.6	1.7
Free phenylalanine pool ($mg \times 10^3$)					
Absolute weight	0.95	1.7	3.0	6.1	8.0
70 kg	1.0	2.3	3.5	8.0	15.5
Distribution quotient*	1.53	0.86	0.80	1.20	0.80
Half-life time† (*min*)	34.2	91.0	177	410	544
Turnover time† (*min*)	49.3	131	255	590	784
Absolute turnover rate ($mg\ phe‡ \times min^{-1}$)					
Absolute weight	19.2	12.7	11.7	10.4	10.2
70 kg	21.2	17.2	13.7	13.6	19.7
$\text{Flux}_{\text{plasma}}$§ ($mg\ phe \times min^{-1}$)					
Absolute weight	1.12	1.24	1.29	0.85	0.84
70 kg	1.24	1.70	1.50	1.11	1.63

* The distribution quotient of free phenylalanine in plasma to the average free phenylalanine concentration in tissue as derived from the equation by Dost (24).

† Values calculated from the first straight line.

‡ phe = Free phenylalanine.

§ Free phenylalanine leaving or entering the intravascular plasma compartment by turnover.

was used instead of phenylalanine, evidence for an (average) extravascular concentration of at least two times that of plasma was obtained by us in one control and in two phenylketonuric patients. Under the assumption that the concentration of α-amino*iso*butyric acid in the total extracellular space is the same as in the intravascular fluid, the concentration of α-amino*iso*butyric acid within the cell is even higher.

To obtain further information on the rapidity of equilibration processes, we have also studied the time required for labeled phenylalanine to be equilibrated *in vivo* between plasma and red cells in Patient K.H. when his plasma phenylalanine level was 10.5 mg per 100 ml. The distribution quotient between red cells and plasma was constant throughout the entire experiment—that is, between 5 and 300 minutes. Since equilibration between plasma and red cells was already complete before 5 minutes after the injection had elapsed, it might not seem unreasonable to assume that all equilibration processes of the labeled phenylalanine in J.I., N.C., and K.H. have essentially come to an end at about 25 minutes, when the first straight line starts. That the uptake of phenylalanine for protein synthesis is also fast will be demonstrated in a second paper (21). Thus, it will be shown that the appearance of a significant amount of labeled plasma proteins in the intravascular compartment can be demonstrated about 30 minutes after the injection of labeled phenylalanine. However, it cannot be excluded that some equilibration is still going on after the first straight line has begun.

If our basic assumption is correct—that equilibration is complete or nearly complete when the first straight line starts and a steady state exists in our patients—then the third component can be best explained by a new process that is showing up. The third component starts in J.I., N.C., and $K.H._1$ at an interval after zero time that is of the order of one turnover time or a little more. After this time, about two-thirds of the old molecules have left the free phenylalanine pool, or only about one-third of the old molecules remain. The return of a small fraction of labeled free phenylalanine from proteins or protein precursor pool to the free phenylalanine pool is, therefore, sufficient to cause significant deviation of the first straight line from its first-order rate. It could also be argued that the appearance of the third component coincides with the end of mixing with slowly equilibrating compartments, which are overburdened with an excessive load of free phenylalanine. It is noteworthy in this regard that the Evans blue space is in a constant relationship with the calculated phenylalanine space—namely, 6 to 11 per cent of it—and that no correlation between plasma phenylalanine levels and phenylalanine space was observed. If some compartments become rate-limiting in the uptake or exchange of free phenylalanine, then it is likely that with increasing plasma phenylalanine concentrations the size of the phenylalanine space decreases, or the time of the intervals between injection of the labeled phenylalanine and the appearance of the first straight line increases, or both. Only the time-interval increase was valid in Patient R.S., with a plasma phenylalanine concentration of 47 mg per 100 ml, while in all the other patients having plasma phenylalanine levels of 2.3, 7.0, 10.5, and 17.0 mg per 100 ml, the time of appearance of the first straight line was essentially the same.

If our basic supposition is false, that equilibration has essentially come to an end when the first straight line starts, then we have to assume that mixing processes are still going on until the third component appears. If the latter view is correct, we have to assume the existence of a dynamically more active and a dynamically less active body compartment, of which our first straight line seems to reflect the first metabolic glycine pool described by Arnstein and Neuberger (30) as well as by Watts and Crawhall (31). The first straight line, then, represents mainly the active body compartment deriving from the fact that dynamically more active organs, such as kidney and liver, have a predominant blood supply and, more likely therefore, a faster equilibration. This is supported by the appearance of a significant amount of labeled protein in the plasma at an early stage. The third component of the disappearance curve would have to reflect complete equilibration between the dynamically more active and the dynamically less active compartment. If this is so, the distribution volume of the dynamically more active and less active compartments would have a larger free phenylalanine distribution volume than has the corresponding water space, indicating significant intracellular enrichment. Calculations from the

final slope of the third component could be used only for estimation of relative turnover rates, since the total phenylalanine pool would be too large, due to processes going on during the time of the first straight line. If this enlarged space were not taken into consideration, the calculated absolute turnover rates would be tremendously high; i.e., 5 and 9 times higher in J.I. and K.H., respectively, than those calculated from the first straight line. In addition, the final slopes possibly do not represent ultimate turnover rates only but also return of labeled material from the proteins or precursor pool, respectively. However, with the data at hand, we feel that the first hypothesis of complete, or nearly complete, equilibration when the first straight line starts is the better model.

All calculations are derived from the first straight line and the values so obtained are summarized in Table I. If our first model is correct, the first straight line represents true turnover as defined above. If the second model should prove correct, the first straight line gives only upper limits for metabolic turnover going on in the dynamically more active body compartment on account of additional unfinished equilibration processes. The plotted curves do not permit a sound resolution into more components owing to the fact that the final slopes are subject to considerable experimental error and, as in Patient N.C., were not observed over a longer period of time.

Our data indicate that phenylalanine concentrations as found in the plasma of phenylketonuric individuals are applicable for inhibition studies in tissue, since the concentration of this amino acid appears to be of the same order of magnitude in plasma and tissue. This discrepancy in Bickis and co-workers' (12) *in vitro* and our *in vivo* experiments, as mentioned earlier, however, could not be resolved on this basis. Perhaps the rate of conversion from tyrosine to *p*-hydroxyphenylpyruvic acid was diminished in the *in vitro* studies, or tissue homogenates and slices per se react differently to phenylalanine.

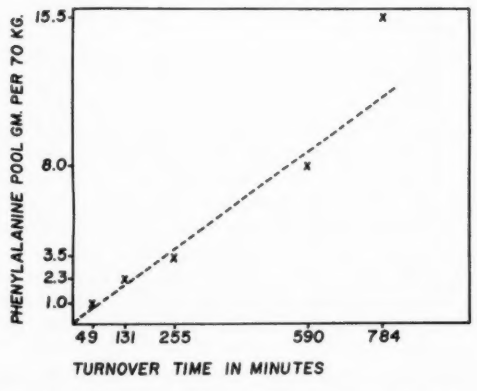


FIG. 2. CORRELATION OF MAGNITUDE AND TURNOVER TIME OF THE FREE PHENYLALANINE POOLS.

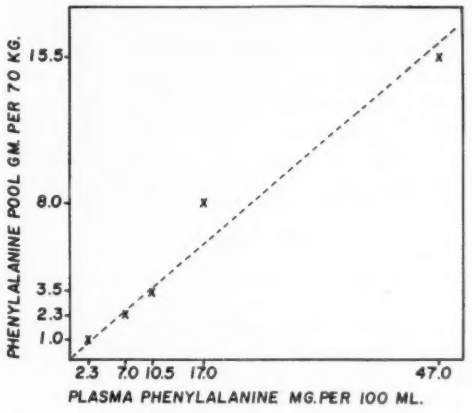


FIG. 3. CORRELATION OF MAGNITUDE OF FREE PHENYLALANINE POOLS AND PLASMA PHENYLALANINE CONCENTRATIONS.

The slopes of the first straight line correlate with the magnitude of the plasma phenylalanine levels; the higher these levels, the smaller the values for the slope constant, and since the slopes of those curves express the turnover time of the free phenylalanine pool, there must be a positive correlation between turnover times and phenylalanine pools. This correlation is illustrated by Figure 2, and the dependence of the correlation between plasma phenylalanine level and free phenylalanine pool is illustrated in Figure 3.

SUMMARY

Four patients suffering from phenylpyruvic oligophrenia were subjected to studies of distribution, pool size, and turnover rate of free phenylalanine. Our data were obtained from the disappearance curves of intravenously injected L-phenylalanine-C^{14}. The experiments indicate no substantial intracellular concentration, as derived

by comparing the phenylalanine space to antipyrine space. A positive correlation existed between the free phenylalanine pool and its turnover time and of phenylalanine pool to plasma phenylalanine concentration. The free phenylalanine pool for normal individuals was estimated to be of the order of 1 g per 70 kg body weight.

ACKNOWLEDGMENT

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CALCIUM METABOLISM OF THE NORMAL RHESUS MONKEY *

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In research related to human nutrition, it has become a tradition first to conduct extensive studies on one or more species of experimental animal, and then to attempt to confirm the findings from these studies on relatively small groups of human beings. It is important that the animal species selected for study should closely resemble the human being with respect to the absorption, metabolism, degradation, and excretion of the nutrient under study. The species acceptable for the study of one nutrient is not necessarily the species of choice for the study of a different nutrient.

The rat and the dog have been used extensively in nutrition studies. These species appear to metabolize many nutrients in a manner similar to that of human beings, but Wanner and co-workers (1) presented preliminary data indicating that neither of these species is a satisfactory substitute for human beings in studies of calcium metabolism. They injected trace amounts of Ca^{45} intravenously, and observed that the ratio of Ca^{45} excretion in the urine and feces during the next 5 days was 1:22 in the rat, 1:10 in the dog, 2:1 in the monkey, and 2:1 in the human being. It has since been reported by Bronner, Harris, Maletskos and Benda (2) that the urine:feces excretion ratios of six young human subjects ranged from 0.50:1 to 3.2:1 and averaged 1.9:1. To our knowledge there are no other reports of similar studies on physiologically normal human subjects. Data obtained on patients with carcinoma, multiple myeloma, and thyroid disorders indicate that the excretion of Ca^{45} is disturbed in these situations, for the urine:feces excretion ratios ranged between 3:1 and 1:3 (3–6). In spite of these anomalies, the "endogenous" excretion of calcium by normal human beings and normal monkeys appears to be similar, and quite unlike that by the dog and the rat.

The data from studies of the calcium metabolism of 29 normal rhesus monkeys (*Macaca mulatta*) are being reported in this communication, and compared with the data from similar studies on human beings conducted by this (2, 7, 8) and by other (3–6, 9–16) laboratories.

EXPERIMENTAL PROCEDURES

All monkeys used in this research were received between 1955 and 1959 from Okatie Farms,[1] N. C. On arrival they were placed in individual monkey cages (27 × 27 × 30 inches) in an air-conditioned (78° F, 60 per cent relative humidity) laboratory designed for metabolic studies with radioactive isotopes. The body weights ranged between 1.8 and 4.9 kg, and all but 3 monkeys were male. The ages of these captive monkeys were not known. Van Wagenen and Catchpole (17) have suggested that a monkey's age may be calculated from his body weight. On this basis these monkeys ranged between 9 and 34 months, with 35 per cent of them just under 2 years old.

During a short period of acclimatization and observation, the monkeys were maintained on a nutritionally adequate diet [2] and distilled water *ad libitum*. All monkeys were fasted overnight before each experiment. Each was then given $Ca^{45}Cl_2$ by intravenous injection, by stomach tube with 200 or 400 mg calcium (as $CaCl_2$) in aqueous solution, or mixed in the diet after it had been

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[1] Maintained by The National Foundation for the quarantine of imported monkeys until cleared of parasites and infectious diseases.

[2] Composition of the maintenance diet (g/100 g): 44.0 farina (unenriched); 22.1 casein (Labco); 16.5 sucrose; 10.0 corn oil (Mazola); 4.0 salt mixture i.v. (18); 3.0 Celluflour; 0.1 *p*-aminobenzoic acid; 0.1 inositol; 0.2 choline chloride; 0.6 ml multivitamin (Vipenta, Roche) drops on alternate days (3 mg thiamine; 3 mg riboflavin; 20 mg niacinamide; 1 mg pyridoxine; 3 mg *d*-pantothenol; 75 mg ascorbic acid; 1 mg *d,l*-α-tocopherol acetate; 2 μg vitamin B_{12}; 5,000 USP U vitamin A; 1,000 USP U vitamin D).

triturated with 120 to 200 mg calcium as the chloride. This labeled meal was offered for 30 minutes and the uneaten residue was analyzed for Ca^{45} and total Ca content. Samples of blood, urine, and feces were collected at appropriate intervals, and analyzed for Ca^{45} and total Ca content. After the dose, blood samples were generally collected after 0.25, 2, 4, and 6 hours; 1, 2, 3, 5, 7 days; and then weekly. Urine and feces samples were collected daily during the first 7 days, and then 48-hour samples were collected at weekly or biweekly intervals until the end of the experimental period.

Twenty-five of the 29 monkeys used in this research were used in subsequent studies of the effects of poliomyelitis paralysis upon Ca^{45} metabolism. Thus, only 4 of the monkeys may be considered as normal control animals at the time of sacrifice. Three monkeys were used in 3 successive experiments in which each received the Ca^{45} by intravenous injection, by stomach tube, and in a labeled meal. Several other monkeys were used in a second experiment after sufficient time had elapsed to permit the Ca^{45} levels in the tissues to stabilize.

At autopsy the gall bladder, heart, liver, kidneys, bone (radius, ulna, humerus, tibia, fibula, femur), and portions of gastrocnemius and biceps muscles were taken from each monkey for analysis. The dissected long bones were roentgenographed.

Samples were analyzed for Ca^{45}, total Ca, moisture, and ash content by the methods outlined by Bronner, and co-workers (8, 19). The data were statistically analyzed, when possible, by methods explained in Snedecor (20); SD in this paper refers to "standard deviation."

The monkeys usually defecated in the corners of the cages, and the stools were sufficiently firm to permit separate collection with little opportunity for the leaching of calcium into the urine. The urine was filtered and rinsed through coarse filter paper to remove food particles.

RESULTS AND DISCUSSION

1. Blood serum data. The total serum calcium content of the 29 monkeys averaged 10.4 mg per 100 ml. The specific activities [3] of the serum cal-

[3] Specific activity = % administered Ca^{45} per mg total calcium in tissue or fluid.

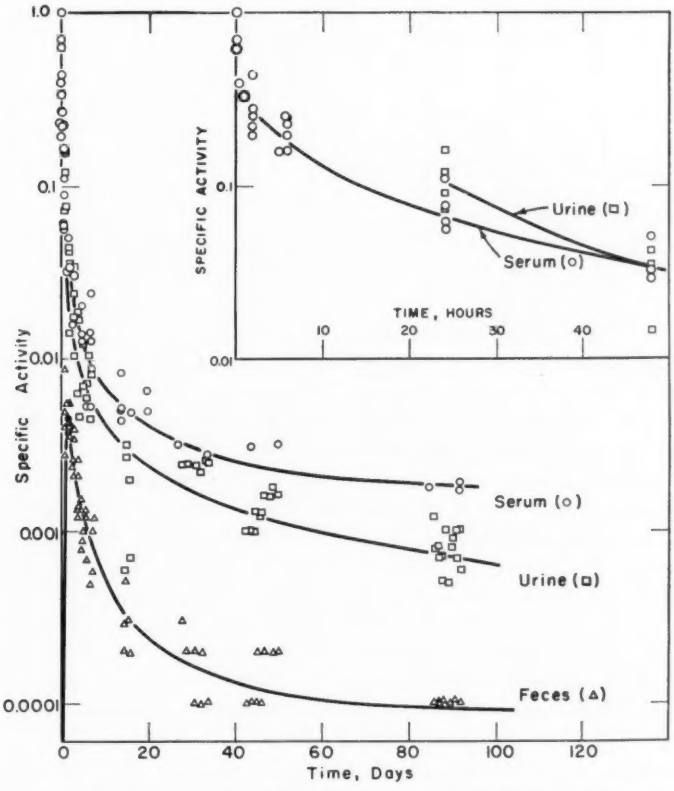

FIG. 1. SPECIFIC ACTIVITIES OF SERUM, URINE AND FECES OF NORMAL MONKEYS AFTER INTRAVENOUS INJECTION OF $Ca^{45}Cl_2$.

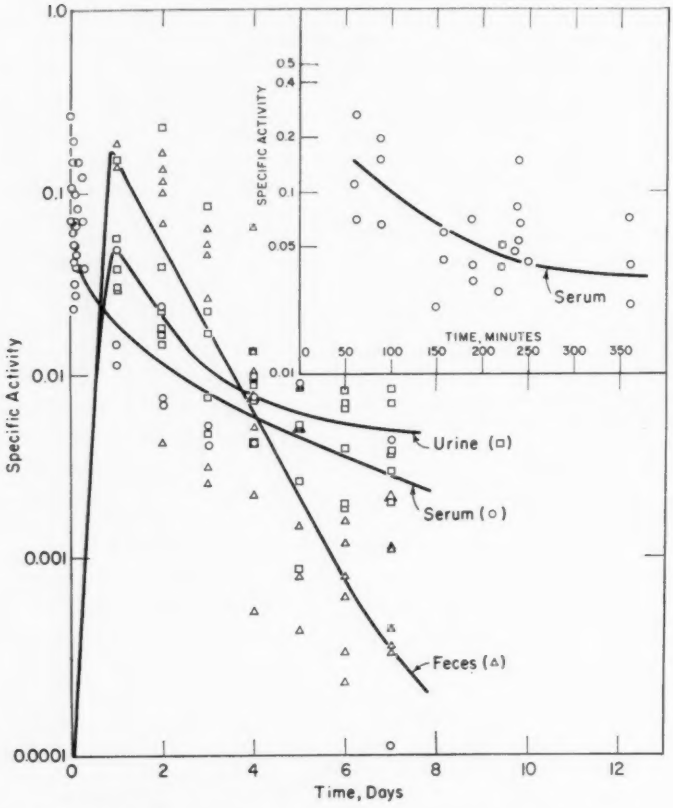

FIG. 2. SPECIFIC ACTIVITIES OF SERUM, URINE AND FECES OF NORMAL MONKEYS AFTER STOMACH TUBE FEEDING OF $CA^{45}CL_2$.

cium samples reached maxima immediately after intravenous injection, approximately 60 minutes after stomach feeding, and 200 minutes after dietary feeding of the Ca^{45} (Figures 1–3). In each case, the activities dropped at decreasing rates during subsequent hours.

The observation that less Ca^{45} was absorbed from the intestinal tract when it was fed in the diet than when given in aqueous solution by stomach tube conforms with the observation by Sharpe, Peacock, Cooke and Harris (21) that food solids interfere with the uptake of radioiron in children. Also, these data are in close agreement with data from similar studies in children, in which the specific activities reached a maximum of 0.6 within 15 minutes of intravenous injection, and a maximum of only 0.2 at 240 minutes after the ingestion of a labeled meal (7). Similarly, in human adults (5) a maximal specific activity of 0.12 was reached 15 minutes after injection, and a maximum of 0.04 was reached about 240 minutes after the ingestion of a labeled meal. Bauer, Carlsson and Lindquist (9) have suggested that the calcium of the body is contained in two fractions: a large relatively inert fraction (99 per cent) contained in the skeletal tissues, and a small amount (1 per cent) of readily exchangeable calcium. The serum specific activity data in Figures 1–3 were used to calculate the exchangeable calcium and the rate of accretion of calcium per day by 24 monkeys by using the method proposed by Bauer and co-workers. The exchangeable calcium, E, was found to be 1.19 g (SD 0.66) or 397 mg per kg body weight in these monkeys. Bauer and colleagues reported a value of 0.782 g exchangeable calcium in an infant weighing 4.3 kg

(182 mg per kg). Heaney and Whedon (14) reported that the exchangeable calcium in adult human beings was less than 100 mg per kg body weight. It appears that the amount of exchangeable calcium varies in relation to the size and age of the subject, being higher in the young child and monkey.

The average accretion rate, A, was calculated to be 0.243 g (SD 0.03) calcium per day in these monkeys, nearly the same as the 0.281 g calcium per day which Bauer and associates calculated for the infant. The accretion rate in the monkeys was about one-tenth that reported by Bronner and Harris (7) for adolescent boys, but the average body weight of the boys was ten times greater than that of the monkeys.

2. Urine data. The Ca^{45} and the total calcium content of the urine recovered from the monkeys which had received Ca^{45} by the three routes are presented in Table I and Figures 1–3. Although the day-to-day excretions of total calcium were varied, the specific activity data were not. As each experiment progressed, the specific activities of the urine and of the serum dropped at approximately parallel rates, when calculated according to the method of Krane, Brownell, Stanbury and Corrigan (5) and Bender (22). This is in agreement with data obtained by us on children (2) which indicated a parallelism between serum and urine specific activity values during 28 days. The peak urinary excretion of Ca^{45} occurred during the first day, whatever the route of Ca^{45} administration.

The total excretion of Ca^{45} in the urine during 5 days after intravenous injection of the radioactive substance averaged 7.56 per cent (SD 7.73). This is in agreement with data obtained by us in similar experiments in children (2) wherein adolescent boys excreted 5.1 per cent of the intravenously injected Ca^{45} within 5 days. The urinary

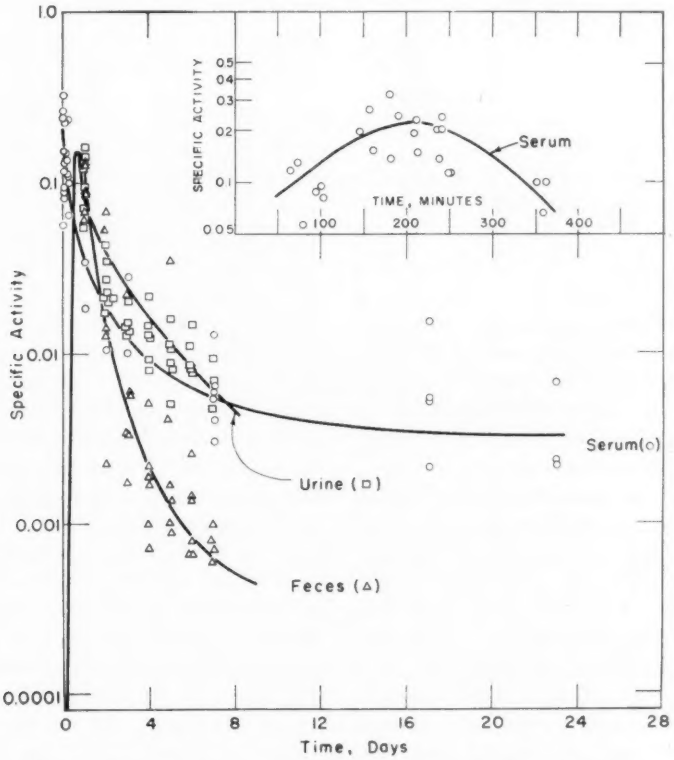

FIG. 3. SPECIFIC ACTIVITIES OF SERUM, URINE AND FECES OF NORMAL MONKEYS AFTER INGESTION OF $Ca^{45}Cl_2$ IN A LABELED MEAL.

TABLE I

Total calcium and Ca^{45} excreted in the urine and feces after Ca^{45} administration to monkeys (averaged values)

Route of Ca^{45} adminis.	Day collected	Urine		Feces	
		Total Ca per day	Ca^{45}	Total Ca per day	Ca^{45}
		mg	*% total dose*	*mg*	*% total dose*
Expt. A Intravenous	0–1	33.8	3.16	221	1.15
	1–2	36.6	1.24	182	0.66
	2–3	52.1	0.90	287	0.73
	3–4	22.0	0.36	210	0.28
	4–5	26.9	0.34	210	0.28
Expt. B_1 Intravenous	0–1	30.7	3.64	210	1.85
	1–2	27.3	1.12	251	1.42
	2–3	31.4	0.74	235	0.80
	3–4	65.5	1.10	206	0.44
	4–5	87.8	1.09	358	0.54
	5–6	75.8	0.55	243	0.29
	6–7	62.5	0.55	317	0.38
	14–15	31.9	0.16	213	0.10
	15–16	46.8	0.15	280	0.08
	27–28	82.7	0.20	112	0.03
	28–29	113.8	0.27	422	0.08
	29–30	113.8	0.26	422	0.08
	30–31	81.4	0.20	327	0.05
	31–32	81.2	0.18	326	0.04
	32–33	87.4	0.23	346	0.06
	33–34	87.3	0.22	345	0.05
	44–45	84.4	0.11	163	0.03
	45–46	84.3	0.10	162	0.02
	46–47	38.1	0.06	214	0.04
	47–48	38.0	0.06	214	0.03
	48–49	45.2	0.08	347	0.08
	49–50	45.2	0.07	346	0.06
	85–86	36.8	0.03	168	0.02
	86–87	37.8	0.03	124	0.01
	87–88	37.7	0.02	124	0.01
	88–89	58.4	0.06	302	0.04
	89–90	58.4	0.05	301	0.03
	90–91	61.7	0.06	210	0.02
	91–92	61.7	0.06	210	0.02
Expt. B_2 Intravenous	0–1	57.0	4.09	204	0.82
	1–2	36.8	0.98	163	0.66
	2–3	41.5	0.42	118	0.30
	3–4	85.3	0.40	247	0.36
	4–5	37.0	0.26	230	0.21
	5–6	51.2	0.31	203	0.20
	6–7	45.4	0.24	380	0.20
	14–15	46.3	0.13	142	0.04
	15–16	78.1	0.16	383	0.09
Expt. C Intravenous	0–1	21.2	1.66	158	0.85
	1–2	28.6	0.41	186	0.67
	2–3	54.5	0.19	173	0.34
	3–4	68.3	0.44	211	0.25
	4–5	63.9	0.46	250	0.21
	5–6	69.8	0.37	250	0.17
	6–7	71.2	0.32	252	0.14
	14–15	49.2	0.03	152	0.03
	15–16	98.1	0.07	166	0.03
	42–43	60.2	0.06	246	0.02
	43–44	130.8	0.14	311	0.02
	44–45	61.0	0.06	162	0.02
	45–46	27.7	0.04	96	0.01
	85–86	51.4	0.06	352	0.02
	86–87	57.8	0.04	387	0.03
	87–88	44.4	0.03	474	0.03
	88–89	36.6	0.02	288	0.02
	89–90	46.1	0.04	222	0.02
	90–91	29.9	0.02	370	0.04
	91–92	107.2	0.06	410	0.02
Expt. D Intravenous	0–1	89.8	13.65	218	0.62
	1–2	96.1	4.27	215	1.13
	2–3	117.4	3.97	269	1.06
	3–4	131.3	2.50	468	1.22
	4–5	121.1	1.72	531	0.88
	5–6	136.5	1.44	293	0.36
	6–7	136.0	1.10	504	0.48
Expt. E_1 & E_2 In diet	0–1	28.8	2.83	188	17.88
	1–2	45.8	1.34	252	7.32
	2–3	37.5	0.63	225	1.74
	3–4	25.9	0.30	169	0.44
	4–5	18.8	0.18	176	1.04
	5–6	29.8	0.26	247	0.32
	6–7	38.8	0.23	250	0.20
Expt. F_1 & F_2 By stomach tube	0–1	52.0	2.72	301*	23.06*
	1–2	24.4	1.32		
	2–3	21.5	0.32	250	7.88
	3–4	35.2	0.38	236	3.10
	4–5	37.4	0.29	320	1.32
	5–6	17.3	0.10	278	0.19
	6–7	27.0	0.15	293	0.16

* Two-day collection was pooled.

excretion data from monkeys are more in agreement with the values for adolescent boys than for adults (2, 13) who averaged between 13 and 20 per cent excretion of Ca^{45} in the urine during 5 days. This may be taken as confirmatory evidence that the monkeys were immature.

The total urinary excretions of Ca^{45} during 5 days by the three monkeys which had received the radioactivity successively by intravenous injection, stomach tube, and in the diet were 6.00 per cent (SD 4.15), 5.02 per cent (SD 4.51), and 5.28 per cent (SD 4.29), respectively. The urinary excretion was significantly higher at the 5 per cent level after intravenous injection than after enteral feeding. This is a reflection of the reduced rate at which the Ca^{45} entered the blood stream due to incomplete uptake of the Ca^{45} after feeding by gastric tube and in the diet.

The significant positive correlation found between body weight and urinary Ca^{45} excretion may be a reflection of differences in the maturity of the bones of these adolescent monkeys. Bronner and Harris (7) observed that an adult man excreted nearly five times as much Ca^{45} in the urine as a teen-aged boy during the 5 days after intravenous injection. The 5-day excretion of Ca^{45} and total calcium in the urine increased as the estimated age of the monkeys increased. This correlation was significant at the 5 per cent level. Similar results have been obtained with mature and aged rats [Hironaka, Draper and Kastelic (23, 24)]. In summarizing the literature on this subject, Harrison (25) presented a graph indicating an inverse correlation between age and calcium absorption.

After the injection of Ca^{45}, the specific activity

of the serum averaged higher than that of the urine (Figure 1).[4] This observation is in accord with the results of Bronner, Benda, Harris and Kreplick (19) who worked with children and with a young man. It agrees in general with the finding of Dow and Stanbury (4), who reported consistently higher specific activity in the urines of patients with thyroid and parathyroid disorders, osteoporosis, and Paget's disease.

Lower specific activities were observed in the sera than in the urine of those monkeys which had received the Ca^{45} by gastric intubation or in the diet.

If the data of Day 1 and Day 7 are assumed to be typical, it is to be noted that the specific activities of the sera were significantly higher (1 per cent level) in those monkeys which received the Ca^{45} intravenously rather than enterally. No significant differences were found in the specific activities of the urines.

3. Feces data. The data on the amounts of Ca^{45} and of total Ca excreted in the feces of the three groups of experiments are presented in Table I and Figures 1–3. Although the daily excretions of calcium were quite variable, the specific activities were rather constant. After intravenous injection the peak excretion of Ca^{45} occurred in 17 of 29 trials on Day 1, in 10 trials on Day 2, and in 1 trial each on Days 3 and 4. After the gastric intubation, the peak occurred on the first day in one trial and on the second day in two of the three trials. Similarly, after the ingestion of Ca^{45} in the diet, the peak excretion occurred on Day 1 in five of six trials, and on Day 2 in the remaining trial. These differences are mainly due to the diet ingested, the peristaltic activity of the intestinal tract, and the frequency of defecation. The passage of Ca^{45} through the intestinal tract appears to have been more rapid in these monkeys than in the human being (2).

The results of 29 trials on 27 monkeys which received Ca^{45} intravenously indicate that the average fecal excretion during 5 days totaled 3.70 per cent (SD 1.76) of the injected dose. Similar data have been reported in studies of adolescent boys who excreted 2.5 per cent of the injected dose, and of one young adult male who excreted 6.8 per cent of the injected dose, within 5 days (7).

The three monkeys receiving Ca^{45} by the three routes in successive experiments excreted 3.09 per cent (SD 0.58), 35.36 per cent (SD 22.13), and 28.43 per cent (SD 9.50) of the administered Ca^{45} in the feces during 5 days after intravenous injection, stomach tube feeding, and dietary ingestion, respectively. The last two values are not significantly different. The results with ingested Ca^{45} in monkeys are in agreement with data obtained in 18 trials on nine adolescent boys, which showed that 24 to 47 per cent of the ingested Ca^{45} was excreted in the feces in 5 days (7). It may be concluded that the high fecal excretion of Ca^{45} after enteral feeding is due mainly to poor absorption.

The average daily excretion of total calcium in the feces after the intravenous injection of Ca^{45} increased with the estimated age of the monkey. This correlation was significant at the 1 per cent level. The total Ca^{45} excretion during the first 5 days also increased significantly (at the 1 per cent level) with the age and weight of the monkey.

Relatively small amounts of Ca^{45} were excreted in the feces when it was administered intravenously, and this excretion occurred mostly during the first few days (Table I). The serum data in Figures 1 to 3 indicate that most of the Ca^{45} taken either intravenously or enterally entered the calcium depots within 1 week. Bronner and coworkers (8) have suggested that studies of the uptake, retention, and excretion of Ca^{45} administered by various routes and in various carriers may be completed within 10 days.

The proportion of the Ca^{45} that was recovered in the feces during 5 days after enteral administration can be used as a measure of the efficiency of Ca^{45} uptake. A small portion of the excreted Ca^{45} represents radioactivity that was absorbed and later re-excreted as "endogenous" calcium. The correction for this endogenous Ca^{45} excretion by monkeys during 5 days is about 3 per cent. Thus the true uptake of Ca^{45} after ingestion in the diet was about 75 per cent. This result is similar to that reported by Bronner and Harris (7) for boys whose uptake varied between 55 and 78 per cent. Blau, Spencer, Swernov and Laszlo (11) reported somewhat lower values of 44 and 67 per cent in two adult patients with carcinoma.

[4] The apparent exception in the Day 1 values is not real, since Fisher's F test showed them not be be significantly different.

Since approximately 35 per cent of the Ca^{45} administered to the monkeys by stomach tube was excreted in the feces, the total uptake was only about 68 per cent. This lower uptake may be a reflection of the emotional stress observed in these monkeys during intubation, and which Malm (26) considered to be a factor in human subjects.

4. Urine : feces ratios of Ca^{45} excretion. The data on the excretion of Ca^{45} in the urine and feces of the 29 monkeys after administration of the Ca^{45} by intravenous injection reveal that an average of twice as much Ca^{45} was excreted in the urine as in the feces during the first 5 days. The same trend continued throughout the experimental period. Thus, the urine : feces ratio of Ca^{45} excretion is 2 : 1 in monkeys, confirming the preliminary report based on only three monkeys (1). The results of these studies in comparison with similar studies on children, adults, rats, and dogs are summarized in Table II. Analyses made in these laboratories indicate that the urine : feces ratio in the human is 2 : 1 (2). Spencer, Li, Samachson and Laszlo (6) reported that the average urine : feces ratio of 5 human females with carcinoma was 2 : 1. Anderson, Emery, McAlister and Osborn (3), reporting therapeutic treatment of a patient with multiple myeloma, gave values which indicate that the urine : feces ratio was about 2.2 : 1. Bellin and Laszlo (10) gave Ca^{45} to two patients with carcinoma. After 7 weeks the average urine : feces ratio was 1.4 : 1. These values resemble those found in normal children and adults (1) but are not like the data reported by Dow and Stanbury (4) from patients who had previously had thyroid disorders but were considered to be euthyroid at the time of study; their urine : feces ratios were much lower, and ranged between 0.6 : 1 and 0.3 : 1. Krane and colleagues (5) studied the calcium excretions of four patients and observed the following urine : feces excretion ratios: one patient with Friedrich's ataxia, 1.5 : 1; one patient with coronary disease, 0.90 : 1; and two patients with treated thyroid disorders, who were considered normal, 1.2 : 1. Since these calcium excretion ratios by patients are quite different from the ratios of normal subjects, it may be concluded that their disorders had produced disturbances in calcium metabolism.

Thus, the monkey resembles the normal human being in relation to the ratios of calcium excreted by the urinary and fecal routes. The rat and the dog are strikingly different in this respect.

5. Tissue data. Four of our monkeys were autopsied and selected tissues were analyzed. The tissue data are summarized in Table III. The radioactivity data are not alike, since the monkeys received different amounts of radioactivity at different times before sacrifice. The specific activities of the soft tissues were not significantly different within each experiment, indicating that the Ca^{45} turnover rates of these tissues were the same; this also was true of the various bones.

TABLE II

Species differences in the excretion of intravenously injected Ca^{45}

Species	Age	Subjects	Condition	Ca^{45} injected	Excreta collected	Excreted	Urine Ca^{45} / Feces Ca^{45} ratios	References
	yrs	*no.*		*μc*	*no. days*	*% dose*		
Human								
♂	12–15	65	Normal	0.7–1.0	5	8	2/1	(2)
♂	21	1	Normal	3.4	5	20	2/1	(7)
♂	adult	1	Multiple myeloma		7	37.4	2.3/1	(3)
♂	65	1	Euthyroid	5	9	24.73	0.6/1	(4)
♀	38	1	Euthyroid	5	9	16.87	0.3/1	(4)
♂	26	1	Friedrich's ataxia	5–7	9	21.17	1.54/1	(5)
♂	50	1	Coronary disease	5–7	9	13.19	0.91/1	(5)
♀	42	1	Euthyroid	5–7	9	26.59	1.16/1	(5)
♀	39	1	Euthyroid	5–7	9	21.74	1.22/1	(5)
♀	59	1	Carcinoma		7	31.0	3.2/1	(6)
♀	52–56	4	Carcinoma		12	23.0	0.8/1	(6)
Monkey	9–34 mo.	29	Normal	1.2–170.2	5	11.3	2/1	This paper
Dog	adult	8	Normal	1.5	5	42.0	1/10	(1)
Rat	120 days	20	Normal	1.0	5	15.0	1/20	(1)

TABLE III

*Ash, Ca^{45} and total Ca of tissues from four normal monkeys after intravenous injection of Ca^{45} **

Tissue	Ash weight		Total Ca		Ca^{45}, % total dose		% Ca^{45} dose/mg Ca†	
	Expt. B	Expt. C	Expt. B	Expt. C	Expt. B	Expt. C	Expt. B	Expt. C
	g	*g*	*mg*	*mg*	%	%		
Heart	0.176	0.152	0.71	0.73	0.0033	0.0010	0.0046	0.0014
Kidney	0.184	0.159	1.10	1.06	0.0050	0.0023	0.0046	0.0022
Liver	1.348	1.523	5.48	3.69	0.0267	0.0046	0.0049	0.0012
Gall bladder	0.048	0.038	0.66	0.61	0.0026	0.0007	0.0039	0.0012
Biceps muscle		0.144		0.78		0.0010		0.0013
Gastrocnemius muscle	0.295	0.239	1.29	0.91	0.0061	0.0012	0.0047	0.0013
Humerus	4.59	4.57	1692	1762	5.46	3.23	0.0032	0.0018
Ulna		2.43		946		1.39		0.0015
Radius		1.89		762		1.30		0.0017
Femur	6.60	7.28	2488	2972	8.41	4.34	0.0034	0.0015
Tibia	4.42	4.89	1584	1890	4.92	2.65	0.0031	0.0014
Fibula		1.05		397		0.40		0.0010
Serum (at autopsy)							0.0050	0.0010

* Monkeys in Experiment B received Ca^{45} at 120 days and again at 3 weeks before sacrifice; those in Experiment C received one injection 13 weeks before sacrifice.

† Specific activity = % administered Ca^{45}/mg total calcium

Experiment B involved two monkeys which had received 170 μc Ca^{45} at 120 days and 133 μc Ca^{45} at 16 days before sacrifice. The specific activities of the bones were significantly (1 per cent level) lower than the soft tissues and the sera. This difference may be a reflection of a slower uptake of the recently injected Ca^{45} by the bones.

Experiment C was concerned with two monkeys which had received 160 μc Ca^{45} 13 weeks before sacrifice. The specific activity of the serum was lower than that of the bones and soft tissues. The specific activities of the soft tissues were significantly (5 per cent level) lower than those of the bones, indicating that once the bones had taken up the radioactivity they released it more slowly than did the soft tissues. The conclusion that there is a difference in Ca^{45} turnover rates of the hard and soft tissues is in agreement with data reported by Bronner and co-workers (19) from a study of a child with gargoylism.

SUMMARY AND CONCLUSIONS

The metabolism of Ca^{45} was studied in 29 rhesus monkeys (26 male, 3 female) maintained on a nutritionally adequate diet. The Ca^{45} was administered by intravenous injection, by gastric intubation, and in the diet. Samples of blood serum, urine, and feces were collected at intervals and analyzed for total Ca and Ca^{45} content. Samples of soft and hard tissues were taken at autopsy and analyzed for Ca^{45}, total calcium and ash content.

Serum Ca^{45} activity reached a maximum within 15 minutes after intravenous injection, 60 minutes after gastric intubation, and 200 minutes after ingestion in the diet.

The average amount of the more readily exchangeable fraction of body calcium was calculated to be 1.19 g per monkey. The accretion rate averaged 0.243 g of calcium per monkey per day.

The excretions of Ca^{45} during 5 days after intravenous injection, gastric intubation, and ingestion in the diet were, respectively: 7.6, 5 and 5.3 per cent in the urine; and 3.7, 35 and 28 per cent in the feces.

The data on the appearance and disappearance rates of Ca^{45} in the sera, the amounts of exchangeable calcium, the amounts of Ca^{45} excreted in urine and feces, and the urine: feces ratios of Ca^{45} excretion reported in this study of 29 monkeys are in close agreement with data previously obtained in similar studies on 65 children. They are in disagreement with data from studies on rats and dogs. The evidence indicates that the monkey is the best substitute for the human being in studies of calcium metabolism.

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PULMONARY DIFFUSING CAPACITY: A COMPARISON OF BREATH-HOLDING AND STEADY STATE METHODS USING CARBON MONOXIDE

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Three methods are in current use for measuring the diffusing capacity of the lungs for carbon monoxide (D_L). Two of these are steady state methods, and they differ in the methods used to calculate the mean alveolar carbon monoxide tension. Filley, MacIntosh and Wright (1) calculate the alveolar P_{CO} indirectly, assuming the physiological dead space for carbon dioxide to be the same as that for carbon monoxide, while Bates, Boucot and Dormer (2) use the end-tidal P_{CO} as the mean alveolar P_{CO}. The third method is the single breath method of Krogh and Krogh (3) as modified by Ogilvie, Forster, Blakemore and Morton (4). Each method in theory measures the total resistance to gas diffusion offered by the structures that lie between the gas in the alveoli and the hemoglobin within the red cells of the pulmonary capillaries. The results obtained by each method differ in normal subjects; and in disease states, such as emphysema, the differences may be great. While the different results obtained in similar subjects by the two steady state methods can largely be explained by the different methods of estimating the mean alveolar P_{CO} (5), the breath-holding method consistently gives higher results than either of the steady state methods (6). Marshall (7) has recently compared the breath-holding method of measuring D_L with a steady state method, using end-tidal samples in a small group of normal subjects and patients with emphysema. He found that, while in normal subjects the end-tidal sample satisfactorily reflected the mean alveolar P_{CO}, in emphysema with impaired intrapulmonary gas mixing, the end-tidal sample overestimates the mean alveolar P_{CO} and therefore gives falsely low results for the diffusing capacity. These results throw doubt on the validity of the end-tidal steady state method of measuring diffusing capacity in subjects with other cardiopulmonary diseases who also have impaired pulmonary gas mixing.

The present study was made *1*) to compare the single breath and steady state methods in a large group of subjects with widely varying intrapulmonary gas mixing; and *2*) since abnormalities of intrapulmonary gas mixing cannot explain the differences that occur between the two methods in normal subjects, a study was made of the variations in the type of breathing which might influence the results of the steady state method in normal subjects.

METHODS

The steady state method used was that of Bates and co-workers (*2*) but the apparatus was modified in a number of details. The valve assembly consisted of single inspiratory and expiratory valves set in 2.5 cm-bore metal tubing in order to reduce the dead space and allow end-tidal samples to be obtained with smaller tidal volumes than were possible with the original apparatus. An automatic end-tidal sampler, triggered by a change in pressure at the mouthpiece, snatched a 35 ml sample at the end of each breath. During the test the subject breathed 0.125 per cent carbon monoxide in air; 1.5 minutes was allowed for the subject to reach a steady state and the expired gas was collected for a further 2 minutes during which time end-tidal samples were taken. The CO concentration in the inspired, mixed expired and end-tidal samples was measured by an infrared analyzer. Oxygen and carbon dioxide in the expired gas were measured by the Haldane method.

The pulmonary capillary carbon monoxide tension cannot be ignored in the steady state method. Subjects with a normal or high diffusing capacity have a considerable increase in blood carbon monoxide during the test, and unless this is allowed for, it may lower the calculated diffusing capacity by as much as 20 per cent (8). Nonsmokers were assumed to have a negligible level of blood CO initially, and measurements of the pulmonary capillary blood CO tension were made only after the tests. In smokers the measurements were made both before and after. We used a modification of the method of

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Sjöstrand (9) which has been described elsewhere (10). The subject breathed oxygen for 3 minutes from an open circuit to wash out the nitrogen from the lungs. He then took a deep breath in and exhaled through a soda lime canister into an empty rubber bag from which he rebreathed for a further 3 minutes. The content of the bag was then analyzed for carbon dioxide, oxygen and carbon monoxide. The carbon monoxide tension of the blood, at the oxygen tension in the lungs during the test, was then calculated from the carbon monoxide and oxygen tensions in the rebreathing bag by the method described by Forster and co-workers (11). The steady state DL is calculated from the equation:

$$DL = \frac{\text{ml CO taken up in 1 minute}}{(ETCO - PBCO) \times (B - 47)}$$

where ETCO is the fraction of CO in the end-tidal sample, B is the atmospheric pressure in mm Hg, and PBCO is the calculated fraction of CO in equilibrium with the pulmonary capillary blood; i.e., CO% in equilibrated bag × calculated end-tidal $O_2\%/O_2\%$ in equilibrated bag.

The measurements on patients at rest, for reasons explained below, were made with the patient hyperventilating to maintain a tidal volume of at least 600 ml (STPD) and a respiratory rate of over 12 per minute. Subjects with tidal volumes or respiratory rates below these limits were excluded from the series. Measurements were made on exercise with the subjects walking on a motor-driven treadmill at a speed to cause moderate dyspnea.

The single breath method used was that described by Ogilvie and colleagues (4). An inspired mixture containing 14 per cent helium, 0.125 per cent carbon monoxide, 20 per cent oxygen and the remainder nitrogen was used. The back pressure of CO in the blood was calculated as for the steady state method. Two estimations of DL were made, with a few minutes between each, and the mean of the two results taken.

Lung volumes and intrapulmonary gas mixing were measured by the helium closed-circuit technique with the apparatus described by Bates and Christie (12). The normal limits of the mixing efficiency index by this method are considered to be 50 to 100 per cent.

Subjects and procedure. The normal subjects were medical students and laboratory staff. All patients attending the laboratory for pulmonary function tests during the period of the study are included in the series; 16 normal subjects and 125 patients with cardiopulmonary disease were studied. The order of investigation follows: *1*) the lung volumes and intrapulmonary gas mixing were measured; *2*) the basal level of CO in the blood was measured in smokers; *3*) two estimations of the single breath DL were made; *4*) the CO in the blood was again measured; *5*) the steady state DL was measured at rest (during this estimation the tidal volume was recorded by a low resistance dry-gas meter on the inspiratory side of the circuit so that tidal volume was immediately read off and the patient was encouraged to increase his tidal volume to about one-third of his vital capacity); *6*) the steady state DL was measured during exercise; *7*) the CO in the blood was again measured.

The steady state DL was also measured in 3 normal subjects at varying tidal volumes both at rest and on exercise and in 4 of the subjects at varying respiratory rates at rest. All volumes are recorded at STPD.

RESULTS

The relationship of steady state DL, ventilation, and exercise in normal subjects. The steady state DL was measured in three normal subjects who breathed at a fixed rate of 12 breaths per minute to a metronome and varied their tidal volumes. The results in all three subjects were similar, and detailed results in Subject GHA at rest are shown in Figure 1. In this subject the steady

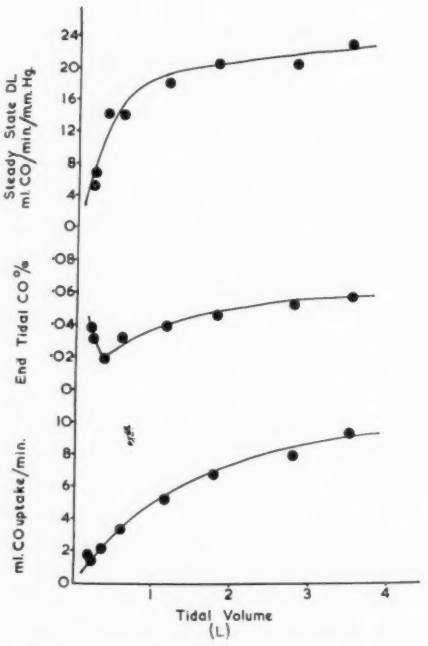

FIG. 1. EFFECT OF TIDAL VOLUME ON STEADY STATE DL, END-TIDAL CO%, AND CO UPTAKE; SUBJECT GHA. Steady state DL measured at a constant respiratory rate of 12 per minute and varying tidal volume. The lower graph shows the steady rise in the volume of CO taken up as the tidal volume increases. The middle graph shows the rise of end-tidal CO as the tidal volume decreases. The rise in CO% at low tidal volumes is due to contamination with dead space gas (CO% of dead space = inspired CO% = 0.125% CO). The quotient of these two, the steady state DL, shows a rapid rise until a tidal volume of about 1 L is reached and after that a slowly rising plateau.

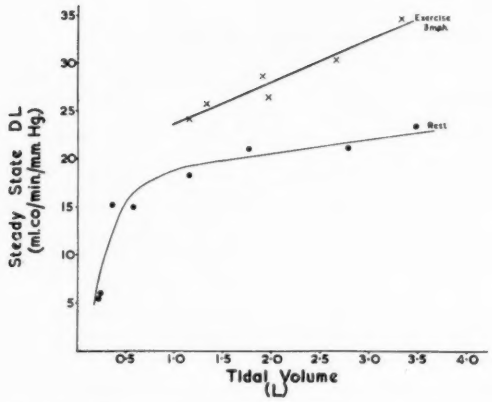

FIG. 2. THE EFFECT OF TIDAL VOLUME ON STEADY STATE DL AT REST AND DURING EXERCISE; SUBJECT GHA. Steady state DL at rest and during steady exercise (3 mph) on treadmill. The subject was breathing at a steady 12 breaths per minute and varying tidal volume.

state DL rose rapidly to reach 15 ml per minute per mm Hg at a tidal volume of 600 ml; thereafter there was a more gradual rise in DL as the alveolar ventilation increased, and at a tidal volume of 3,700 ml the DL was 23 ml per minute per mm Hg. The volume of CO taken up per minute increased from 2 ml per minute when the tidal volume was a few hundred ml to about 9 ml per minute at the greatest tidal volume. The increase in end-tidal CO per cent and the fall in DL at low tidal volumes are due to contamination of the end-tidal sample with dead space carbon monoxide. From the results of these experiments it was concluded that a tidal volume of 600 ml was the minimum necessary for end-tidal sampling, and the steady state DL varied with the alveolar volume or alveolar ventilation at which it was measured.

A similar rise of DL with increasing tidal volume was also found during exercise. Two subjects exercised at a steady speed of 3 mph and again breathed to a metronome at 12 breaths per minute. The results in two subjects (GHA and RM) were very similar and Figure 2 shows the results in Subject GHA. The most comfortable tidal volume at this respiratory rate was about 0.5 L at rest and about 2 L on exercise. The difference between DL measured during quiet breathing at rest (DL = 15 ml/min/mm Hg with a tidal volume of 0.5 L) and that on exercise (DL = 27 ml/min/mm Hg with a tidal volume of 2.0 L) is 12 ml per minute per mm Hg. Of this increase, 5 ml per minute per mm Hg can be produced at rest by increasing the tidal volume to 2.0 L; the other 7 ml per minute per mm Hg is presumably the result of exercise.

In a second series of experiments nine normal subjects were examined, and both the steady state and single breath DL measured. The steady state DL was measured at rest during "quiet" breathing, during hyperventilation at the same respiratory rate (11 per minute) and during exercise. The tidal volume during the "quiet" respiration was at least 600 ml, and during hyperventilation it was as deep as possible. During exercise the subject breathed at his most comfortable rate and

TABLE I

Comparison of steady state DL measured during quiet breathing and hyperventilation with single breath DL in 9 normal subjects all breathing at rest to a metronome set at 11 breaths per minute *

						Steady state DL									Fraction Steady state DL		
						A. Quiet breathing			B. Hyperventilation			C. Exercise			Single breath DL		
Subject	Age	Sex	SA	Vital capacity	Single breath DL	VT	Resp. rate	DL	VT	Resp. rate	DL	Speed	VT	Resp. rate	DL	A	B	C
			m^2	ml								mph						
RM	34	M	1.95	4,540	34.7	865	10	22.0	2,510	10	29.0	3.5	1,060	21	32.6	0.64	0.84	0.94
GHA	34	M	1.95	4,300	31.0	660	10	17.1	3,500	12	24.0	3	1,045	17	31.4	0.55	0.78	1.01
JSM	22	M	1.65	3,468	36.2				1,738	10	33.0	3	760	24	42.6		0.91	1.18
RDW	25	M	2.31	5,730	28.9	1,052	10	20.0	2,995	10.5	32.3	3	1,705	14	34.0	0.69	1.12	1.18
CPR	21	M	1.93	5,875	56.0	795	12.5	32.6	3,450	10	53.7	3	1,540	16	46.0	0.58	0.96	0.82
JAW	20	F	1.61	3,275	29.2	640	12	20.3	1,880	12	24.5	2.5	757	15	25.4	0.70	0.84	0.87
MEU	33	F	1.74	3,500	27.5	993	11	19.0	1,918	11	27.3	2.5	1,520	14	35.7	0.69	0.99	1.31
MC	22	F	1.50	2,900	24.0	1,070	11	23.1	1,921	11	27.0	3	1,420	9.0	37.0	0.96	1.13	1.54
PJB	32	M	1.97	4,500	34.0	505	13	26.8	1,766	11	32.4	3	1,382	14	43.6	0.79	0.95	1.28
Mean figures	27		1.85	4,220	33.5	822	11	22.6	2,330	10.7	32.0	3	1,243	16	36.5	0.70	0.95	1.1

* SA = surface area in square meters; VT = tidal volume in ml; resp. rate = respiratory rate; A = during quiet breathing at rest; B = during hyperventilation at rest; C = during exercise. All volumes measured STPD.

TABLE II

The effect of increase in respiratory rate at constant tidal volume on steady state DL

Subject	Sex	Age	Surface area	Respirations	Tidal volume	Minute volume	Steady state DL
			m^2	*rate/min*	*ml*	*L/min*	*ml CO/min/ mm Hg*
RM	M	34	1.95	10	865	8.6	22.0
				20.6	850	17.5	22.5
				26.3	850	22.4	20.0
GHA	M	34	1.95	9.5	2,150	20.2	33.0
				15.0	2,500	37.6	25.0
				25.5	2,060	52.6	25.3
RW	M	26	2.30	8.0	2,290	17.7	52.0
				17.4	2,170	37.8	46.0
				24.3	2,270	55.1	45.0
MH	F	30	1.69	10.2	1,100	11.2	22.2
				18.6	1,270	23.6	17.3
				31.4	1,260	39.5	18.9

tidal volume. The results of the experiments are shown in Table I. A rise in the steady state DL occurred in all subjects during hyperventilation, and during hyperventilation the steady state DL and the single breath DL became approximately equal. During quiet breathing the mean steady state DL was 70 per cent of the mean single breath DL, while during hyperventilation at the same respiratory rate the steady state DL was 95 per cent of the single breath DL. The steady state

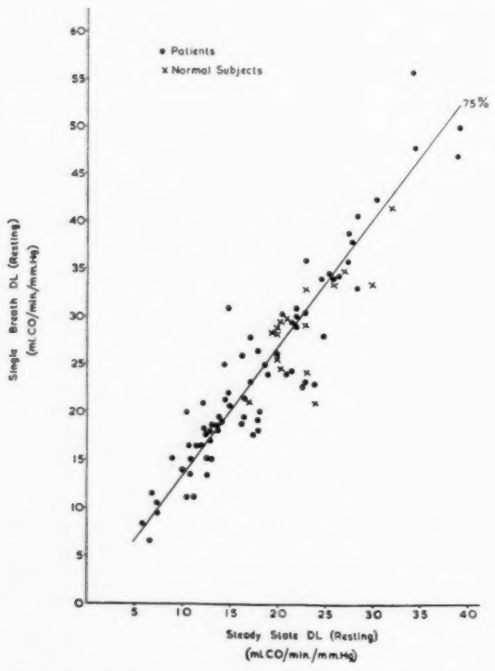

FIG. 3. STEADY STATE DL PLOTTED AGAINST SINGLE BREATH DL IN SUBJECTS WITH NORMAL INTRAPULMONARY GAS MIXING. Mean relationship 75%.

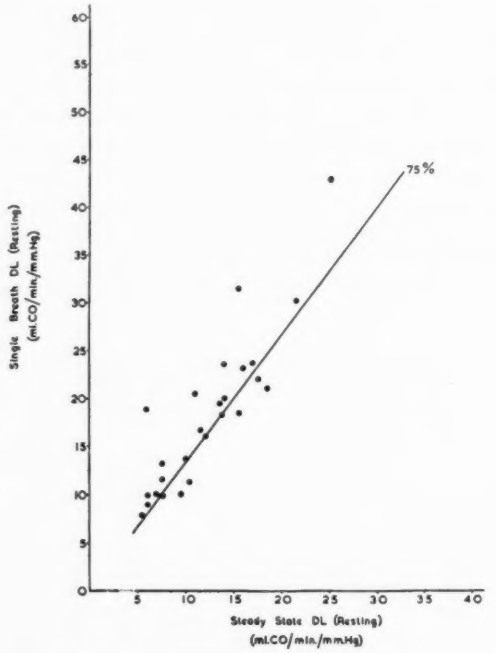

FIG. 4. STEADY STATE DL PLOTTED AGAINST SINGLE BREATH DL IN PATIENTS WITH IMPAIRED MIXING BUT EXCLUDING THOSE WITH EMPHYSEMA. The line indicating the 75% relationship which exists in normal subjects is shown for comparison.

TABLE III

The relationship between steady state DL and single breath DL in subjects with a normal mixing efficiency, patients with impaired mixing but no emphysema, and patients with emphysema

Group	Subjects	Helium mixing index within range	Mean helium mixing	Fraction Steady state DL / Single breath DL		Mean rate of exercise	Mean oxygen consumption during exercise
				Resting	Exercise		
	no.	*%*	*%*			*mph*	*ml/min*
1. Normal subjects	16	50–100	83	0.72 } 0.75	1.09	2.9	1,050
Patients with normal mixing	74	50–100	71	0.74 }		2.0	913
2. Patients with impaired mixing but no evidence of emphysema	26	0–49	36	0.66	0.91	1.7	766
3. Emphysema	25	0–49	29	0.34	0.41	0.8	714

DL on exercise was higher than during hyperventilation at rest in all but one case.

In four subjects the steady state DL was measured at a steady tidal volume and varying respiratory rates. The diffusing capacity was not related to the rate of breathing or the minute volume. The results are summarized in Table II.

A comparison of steady state and single breath methods in patients with cardiac or pulmonary disease. The 125 patients and 16 normal subjects were divided into three groups.

Group 1, subjects with a mixing efficiency of 50 per cent or more: 16 normal subjects and 74 patients.

Group 2, subjects, excluding those with chronic obstructive emphysema, whose mixing efficiency was below 50 per cent: 26 patients.

Group 3, subjects with a clinical diagnosis of chronic obstructive emphysema: 25 patients, all with a mixing efficiency of less than 50 per cent.

The details of the composition of each group and the mean figure for intrapulmonary gas mixing are shown in Table III, together with the mean oxygen consumption at rest and during exercise, and also the rate of exercise. Groups 1 and 2 were largely composed of patients with rheumatic heart disease, congenital heart disease and sarcoidosis but included some patients with thyrotoxicosis and some with asthma. The lowest DL's were recorded in patients with emphysema or with alveolar-capillary block syndrome (13) due to sarcoidosis, metastatic carcinoma or idiopathic pulmonary fibrosis. The highest figures were obtained in patients with congenital heart disease with increased pulmonary blood flow. Most of these patients had atrial septal defects with left to right shunts. All the patients in group 3 suffered from chronic obstructive emphysema. The diagnosis was made on the clinical and radiological findings before they were referred for respiratory function studies.

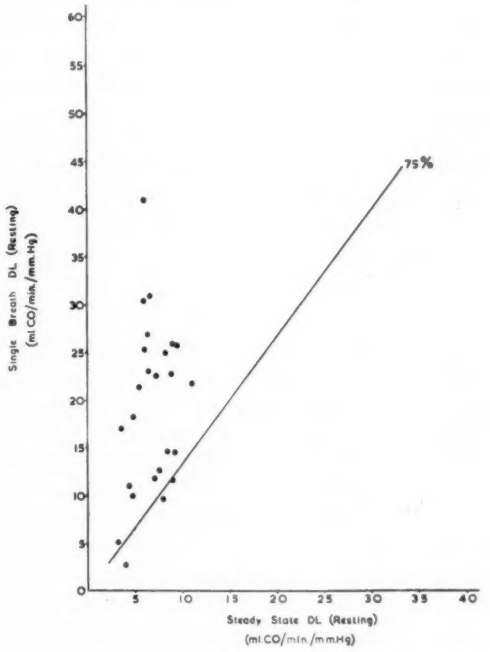

FIG. 5. STEADY STATE DL PLOTTED AGAINST SINGLE BREATH DL IN SUBJECTS WITH EMPHYSEMA. The line indicating the 75% relationship which exists in normal subjects is shown for comparison.

The single breath DL was plotted against the steady state DL separately in each group, and the results at rest are shown in Figures 3, 4 and 5, and during exercise in Figures 6 and 7. The scale is identical in each case. The relationship between the two measurements of DL was calculated by the method of least squares. Figure 3 shows the results in group 1, which was composed of all subjects with normal intrapulmonary gas mixing. The steady state figure was 78 per cent of the single breath figure for the group of 16 normal subjects, and 74 per cent for the 74 patients with normal intrapulmonary gas mixing. The mean figure for the whole group was 75 per cent. In group 2, which includes all patients with impaired intrapulmonary gas mixing, but with no clinical evidence of emphysema, the mean steady state DL was 66 per cent of the single breath DL (Figure 4).

The mean pulmonary gas mixing index of group 3 (29 per cent) is comparable with that of group 2 (36 per cent), but in group 3 the single breath and steady state methods give results that bear no relationship to each other (Figure 5). All the patients with emphysema showed a steady state DL below 10 ml per minute per mm Hg, while the corresponding single breath DL varies between 3 and 42 ml per minute per mm Hg. The finding that patients with clinical emphysema have a steady state DL below 10 ml per minute per mm Hg is in agreement with the results of Bates, Knott and Christie (14) who used the same method.

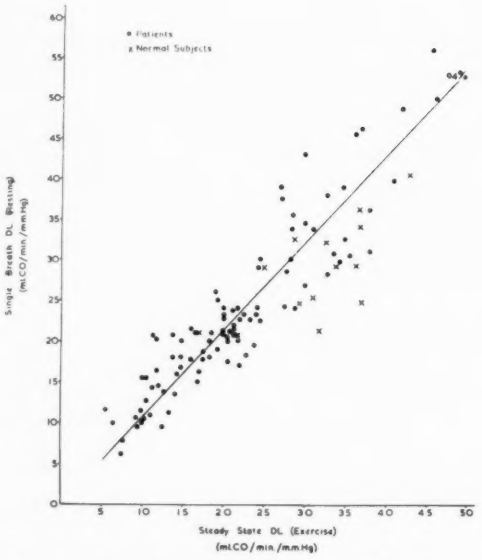

FIG. 6. STEADY STATE DL DURING EXERCISE PLOTTED AGAINST THE RESTING SINGLE BREATH READING. Mean relationship 94%. The graph contains all subjects except those with emphysema.

DISCUSSION

A rise in steady state DL was found when normal subjects hyperventilated. This is in line with previous reported work. Ross, Frayser and Hickam (15) and Turino, Brandfonbrener and Fishman (16) using the Filley method, and MacNamara, Prime and Sinclair (17) using the Bates method of measuring the steady state DL, all found a rise in DL when subjects hyperventilated. In this series, when normal subjects hyperventilated by progressively increasing their tidal volume while keeping the respiratory rate unchanged, a progressive rise in DL occurred (Figure 1); but when the subjects hyperventilated by progressively increasing the respiratory rate while keeping the tidal volume steady, no rise in DL occurred (Table II). This suggests that the DL varies with the alveolar volume at which it is measured rather than with the alveolar ventilation.

There seems little doubt that DL measured by the single breath method also varies with the alveolar volume. Although Ogilvie and colleagues (4) found DL varied little at different alveolar volumes, Marks, Cugell, Cadigan and Gaensler (6) and Shephard (18) found DL at full inspiration to be up to 50 per cent greater than DL measured at the functional residual capacity, while McGrath and Thomson (19), under similar circumstances, found a mean increase in DL of 31 per cent in eight normal subjects. In a previous study Marshall (7) measured the single breath DL at varying alveolar volumes and showed that DL decreased in such a way that, when the breath was held at the functional residual volume, the single breath DL became approximately equal to the steady state DL using end-tidal samples.

In this series there was good agreement between the resting steady state DL measured at maximal tidal volume and the resting single breath DL (Table I). These findings suggest that there is no fundamental difference between the two methods in normal subjects. The increase in DL on hyper-

ventilation may be due to an increase in the pulmonary capillary blood volume in association with an increased alveolar gas volume and, therefore, a larger blood gas interface. This suggests that the resting single breath DL measured at maximal inspiration and the resting steady state DL measured on maximal hyperventilation are equal, and both reflect the maximal diffusing capacity of the lungs for carbon monoxide in the resting subject.

The steady state DL on exercise was higher than the hyperventilation DL in all but one subject, and higher than the single breath DL in six of the nine subjects. Although the exercise DL obtained in these subjects was not usually equaled during hyperventilation or when measured by the single breath method, the mean exercise DL was only 11 per cent greater than the resting single breath DL. Ross (15), and Turino (16), and their associates made observations at rest and during exercise, using the Filley method. They showed that in the series as a whole, the increase in DL on exercise was no greater than that produced by an equivalent degree of hyperventilation at rest. In the present study, repeated measurements on three subjects (the results on one of whom are shown in Figure 1) have shown that exercise produces an increase in DL, measured by the steady state method, which is greater than that which can be accounted for by the hyperventilation alone. This is in keeping with the findings of MacNamara and co-workers (17) who also used the Bates method of measuring DL.

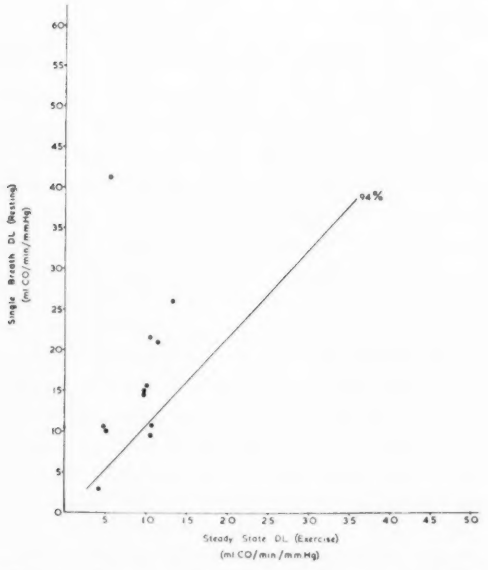

FIG. 7. STEADY STATE DL DURING EXERCISE PLOTTED AGAINST THE RESTING SINGLE BREATH DL IN 12 CASES OF EMPHYSEMA. The line indicating the 94% relationship which existed in all other subjects is shown for comparison.

The diffusing capacity is not a constant but varies with the alveolar volume at which it is measured, and it is clearly important to control the tidal volume at which DL is measured if the results are not to be misleading. In this context the single breath method has the advantage that the alveolar volume at which it is measured is easily fixed at maximal inspiration, while hyperventilation in the untrained subject at rest may result in irregular tidal volumes and an unreliable figure for DL. Measurement of the steady state DL during exercise overcomes this difficulty, but the result must then be related to the rate of work (2) or oxygen consumption (20) and functional residual volume (2) or surface area.

The general relationship between the steady state and single breath DL in this series of 141 subjects is similar to that obtained by Marks and co-workers (6) who found the Filley steady state method gave results about two-thirds of the single breath DL.

The patients in group 1, with cardiac or respiratory disease but normal intrapulmonary gas mixing, behaved similarly to the normal subjects, the steady state DL being equal to approximately 75 per cent of the single breath DL (Figure 3).

The difference in the ratio of steady state DL to single breath DL in group 1 (74 per cent) compared with group 2 (66 per cent) may be due to a number of factors. Patients in group 2 were more incapacitated than those in group 1 (Table III) and breathed at a smaller tidal volume. Borderline cases such as one with severe persistent asthma and widespread cystic bronchiectasis (steady state DL 4.6, single breath DL 18 ml CO/min/mm Hg) were included in group 2 rather than 3. In addition, it is likely that in the presence of impaired intrapulmonary gas mixing the steady state DL is too low because of end-tidal sampling errors. This point is discussed further below, but the maximal effect of inaccurate end-tidal sampling in group 2 is to lower the steady state DL by only 10 per cent.

The difference in the ratio of D_L calculated by the two methods in group 2 (patients with impaired intrapulmonary gas mixing) and group 3 (patients with emphysema) is more interesting. Although the helium mixing index is similar in both groups, the steady state and single breath D_L bear no relation to each other in patients with emphysema. The reasons for this may be:

1. The single breath D_L is too high in these patients. The single breath D_L is calculated from the alveolar volume obtained by adding the inspired volume to the residual volume measured by the helium equilibration technique. In patients with unequal gas mixing this volume (V_{eq}) is greater than that calculated from the dilution of helium inspired in the single breath test (V_{sb}). The alveolar volume V_{sb} is only an approximate measure of the volume ventilated by the single breath, because the expired sample collected may not represent the mean helium concentration in the lungs; the highest concentrations of helium are discarded with the dead space gas, and the less well ventilated parts of the lungs contribute only a small volume of the collected sample. In practice, the single breath D_L calculated from V_{sb} is reduced to 95 per cent of the normally calculated single breath D_L in patients in group 2. The patients with emphysema (group 3) have a mean single breath D_L calculated from V_{sb} which is 70 per cent of the normal single breath D_L. Even when this reduced single breath D_L is taken into consideration, there is no relationship between the single breath and steady state D_L in group 3.

A further possible cause of error, giving falsely high results by the single breath method in patients with emphysema, lies in the length of time taken to collect the alveolar sample. In this series the breath-holding time was measured from the beginning of inspiration to the start of sample collection (4). By recalculating D_L, using "effective breath-holding time" as suggested by Jones and Meade (21)—namely, from the beginning of inspiration to the endpoint of sample collection, less 3/10 of inspiration time—D_L dropped by an average of 10 per cent, but there was still no relationship between the single breath and steady state methods in this group.

2. The steady state D_L is too low. Marshall (7) has recently discussed the end-tidal sampling method of measuring D_L in patients with emphysema. In emphysema, where no clear division exists between dead space and alveolar gas, the end-tidal samples contain more CO than the mean concentration of CO in the lungs. The steady state method using end-tidal samples therefore gives falsely low figures for the over-all diffusing capacity of the lung. Bates (22) believes the steady state method with end-tidal sampling reflects the diffusing capacity of the predominantly ventilated portion of the lung.

The most striking difference between groups 2 and 3 is the lack of correlation between the single breath and steady state D_L either at rest or on exercise in group 3. Although the mean mixing efficiency index of group 3 (29 per cent) is only slightly lower than that of group 2 (36 per cent), this index is not a sensitive measurement of gas mixing and the differences of gas distribution in the two groups may be greater than that indicated by the mixing index figures. If the ratio V_{sb}/V_{eq} is used as a measure of mixing efficiency, a much greater difference is found between the two groups. A lower mixing efficiency in group 3 than in group 2 would contribute to the lack of correlation between the two methods in these groups.

3. The main cause of the differences between groups 2 and 3 is probably the inequality of ventilation and perfusion in the emphysematous lung. The volume of unperfused or underperfused lung of emphysema patients is increased, and this increased physiological dead space is ventilated mainly by the tidal volume. When the D_L is measured with normal tidal volumes, as in the steady state method, a large proportion of the tidal volume will ventilate physiological dead space and give a low D_L. A previous study by Marshall (7) showed that the D_L in emphysema was uniform throughout most of a full expiration. The method was not capable of detecting changes in the first few hundred ml of alveolar gas air, and it is probable that the gas first expired, which has the highest ventilation-perfusion ratio, comes from regions of the lung with a low D_L. High values for the single breath D_L with low values by the steady state method could occur when the pathological changes in the lungs are such that the most severely affected parts of the lung are ventilated by the tidal volume and when the re-

mainder of the lung is less severely affected. When the emphysematous changes in the lungs are diffused throughout the lungs, both the single breath and steady state DL will be low.

It appears that in the absence of emphysema either method gives a satisfactory figure for DL. The difference between the results obtained by the two methods can largely be accounted for by the different alveolar volume at which they are measured, although impaired intrapulmonary gas mixing may lower the steady state result by approximately 10 per cent.

In emphysema neither method measures DL satisfactorily. The steady state method gives consistently low results for reasons discussed above and, although this makes the steady state method a useful test clinically, the results do not reflect the true diffusing capacity of the lungs. The single breath method gives results in emphysema which are often optimistic and which, although they may be of value in investigating the physiology of the disease, are usually of little help in the assessment of disability.

SUMMARY

In 16 normal subjects and 125 patients with cardiac or respiratory disease, measurements of the diffusing capacity of the lungs for carbon monoxide (DL) were made by both the single breath and the steady state methods with end-tidal samples as a measure of alveolar CO.

In 16 normal subjects at rest the steady state DL was about 75 per cent of the single breath DL. The steady state DL increased with increasing tidal volume so that at maximal tidal volume the steady state DL was approximately the same as the single breath DL. The increase of DL on exercise was greater than could be accounted for by the increased ventilation on exercise. An increase of respiratory rate without an increase in tidal volume did not increase DL.

The patients with cardiac or respiratory disease were considered in three groups. Group 1 (74 patients with normal pulmonary gas mixing) : the steady state DL was 74 per cent of the single breath DL at rest. Group 2 (26 patients with impaired pulmonary gas mixing but no clinical evidence of emphysema) : the steady state DL was 66 per cent of the single breath figure. The difference between groups 1 and 2 may be due, in part, to the errors in end-tidal sampling in patients with uneven pulmonary ventilation. Group 3 (25 patients with emphysema) : there was no relationship between the single breath and steady state DL. The reasons for this are discussed.

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CARDIORESPIRATORY EFFECTS OF EXPERIMENTAL LUNG EMBOLISM *

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Respiratory embarrassment and pulmonary hypertension are important consequences of pulmonary embolism. Respiration is affected at several levels. Changes in rate and depth have been summarized by Whitteridge (1) as "rapid shallow breathing" unaffected by the inhalation of 100 per cent oxygen (2, 3). Sectioning or cooling of the vagi prevented this phenomenon which was therefore regarded as neurogenic, caused by the excitation of (unspecified) lung receptors.

Embolism is usually followed by an increased resistance to lung inflation in the artificially respired animal (4, 5) or a rise in the intrapleural pressure swing in those breathing spontaneously (6). This was thought to be due to bronchoconstriction.

A fall in arterial oxygen saturation has also been reported to accompany pulmonary embolism; several mechanisms were suggested to explain this change. An acceleration of blood flow through the non-occluded areas of the lung (= decreased contact time) was proposed by Binger, Brow and Branch (2). A fall in the pulmonary diffusing capacity for oxygen was assumed by Williams (7) to be due to a reduction in the area of the alveolocapillary membrane. Venous admixture was not abolished, however, by breathing 100 per cent oxygen (8, 9), and it also occurred after the intravenous injection of oxygen itself (10). This prompted the conclusion that arterial hypoxia was caused by the opening of pulmonary arteriovenous anastomoses (9).

These changes do not seem to account for the often distressing dyspnea seen in clinical pulmonary embolism. It occurred to us that changes in lung mechanics—the most common causes of dyspnea—have not yet been investigated in this condition, and we therefore decided to include lung mechanics in our study of experimental lung embolism.

The dose-response relationship in embolic pulmonary hypertension, as demonstrated by several investigators, suggests that the rise in pressure is related to the number of occluded vessels. This implies that pulmonary hypertension would occur only in widespread embolization, since a 50 per cent occlusion of the pulmonary circulation is followed by mild changes only (Table I). Two-thirds of the pulmonary vascular bed had to be excluded in order to create a more marked pulmonary hypertension (14, 15).

These data are difficult to reconcile with some other observations. Repeated intravenous injections of blood clots into rabbits resulted in a marked right ventricular hypertrophy, suggesting pulmonary hypertension. Yet postmortem angiography of the lung vessels failed to reveal obstruction (16). This finding and numerous others not mentioned here appeared to indicate that reactive vasoconstriction contributes to this type of pulmonary hypertension. Embolic constriction of the systemic arteries was shown to be neurogenic. It was expected that neuroplegic procedures might also modify the pulmonary vascular response to embolism, but in the majority of technically acceptable experiments, spinal cord section, vagotomy, and chemical and surgical sympathectomy were found ineffective (7, 17–19). Only Price, Hata and Smith (20) obtained a reduced response.

It was decided to study two aspects of these changes. By using two different doses of embolic material (one 10 times larger than the other), an attempt was made to assess the quantitative nature of the response. By using different drugs,

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† Adolph Basser Research Fellow of the Royal Australasian College of Physicians.

‡ Joint Coal Board Research Fellow.

TABLE 1

Changes of pulmonary circulation with pulmonary vascular occlusion *

Subjects	Pulm. art. mean press. C	Pulm. art. mean press. O	Cardiac output C	Cardiac output O	Ventil. C	Ventil. O	Pulm. art. resist. C	Pulm. art. resist. O	Ref.
	mm Hg		*L/min*		*L/min*		*dynes-sec-cm⁻⁵*		
5 men	16	23	7.9	8.2			162	224	(11)
15 men	13	15	9.3	8.4	8.6	8.3	112	143	(12)
9 dogs	15	18	3.1	3.1	3.2	4.3	387	464	(12)
6 men	21	30	5.8	5.5			290	435	(13)

* C = control; O = occlusion.

the possibility of a non-neurogenic pulmonary vasoconstriction was explored.

METHODS

Material. Forty-six sheep weighing 21 to 48 kg were used in these experiments. The supine animals were anesthetized with thiopentone and intubated. The femoral artery and vein were dissected free and a thermometer was inserted into the rectum.

Circulation measurements. A cardiac catheter was passed via the femoral vein into the pulmonary artery and a cannula was introduced into the femoral artery. Oxygen saturation of the arterial (Sa_{O_2}) and mixed venous ($S\bar{v}_{O_2}$) blood and hemoglobin content were measured spectrophotometrically (21). In experiments in which 100 per cent oxygen was breathed instead of air, oxygen content and capacity were measured manometrically (22). Expired air was collected for 1.5 to 2.5 minutes in Douglas bags; their content was measured and analyzed by the Haldane method. In later experiments oxygen uptake, tidal volume (V_T) and rate of breathing (f) were measured from the record obtained with a Pulmotest twin-spirometer.[1] The animal breathed air from a closed-circuit system, oxygen being fed from the second bell at the rate at which it was removed from the first system. The same arrangement was used for experiments performed with 100 per cent oxygen, both systems being washed out and filled with oxygen.

Blood samples were taken in the mid-period of ventilation measurements. The Fick principle was used to calculate pulmonary arterial blood flow ($\dot{Q}$), the shunt-formula ($Cc_{O_2} - Ca_{O_2}/Cc_{O_2} - C\bar{v}_{O_2}$) to calculate venous admixture (Q_s) in per cent of $\dot{Q}$. Oxygen content of the pulmonary capillary blood (Cc_{O_2}) was estimated by subtracting 0.60 vol per cent from the oxygen capacity of the arterial blood (23). In other experiments (24) this method gave good agreement with the more laborious procedure of calculating alveolar oxygen tension, measuring blood pH and obtaining the saturation of pulmonary capillary blood from the oxygen dissociation curve for sheep's blood.

Pulmonary ($P_{p.a.}$) and femoral ($P_{f.a.}$) arterial pressures were measured by Sanborn transducers (no. 267) and recorded on a 6-channel direct-writing Sanborn oscillograph and expressed in millimeters of mercury. Total pulmonary (R_{pulm}) and total systemic (R_{syst}) resistances were calculated by the usual formulas and expressed in dynes-sec-cm^{-5}.

Blood flows, resistances and ventilated volumes were expressed on the basis of 1 m^2 body surface area (BSA). The latter was calculated by the formula: $8.3\sqrt[3]{w^2}$ where w = body weight in grams (25). Details of the circulation measurements were described elsewhere (26).

Intrapleural pressure (P_{pl}) was measured via a specially prepared 17 gage needle introduced into the third or fourth interspace, 7 to 10 cm from the anterior surface of the chest. A pneumothorax of 60 to 80 ml was induced. The needle was connected by 70 cm polyethylene tubing to a Sanborn transducer no. 267 B. P_{pl} was recorded relative to atmosphere in centimeters of water.

Air flow rate and tidal volume were obtained with a Godart pneumotachometer and integrator.[1] The pneumotachometer is similar to that described by Silverman and Whittenberger (27) and the one used in these experiments covered the range of flow from 0 to 100 L per minute. The integrator consists of a high stability D.C. amplifier with an extremely effective time constant and a low drift rate so that the error of V_T measurements from this source was not likely to exceed 1 per cent. The P_{pl}, airflow and volume changes were electrically amplified and recorded simultaneously on the oscillograph.

Method of analysis. Lung compliance (C_L), expressed in milliliters per centimeter water per kilogram, was obtained by dividing the V_T by the simultaneously recorded P_{pl} between points of zero flow; 6 to 10 breaths were measured from a recording made at 25 to 50 mm per second and the mean value taken. Mean frictional (= nonelastic) resistance was calculated at points of equal volume (28). Differences in pressure and rate of airflow were read from the recording. Due allowance was made for the resistance of the instrument. Five breaths were analyzed for each period.

Calibration. Pressure, volume and flow were all linear within 2 per cent. The coefficient of variation for a single measurement of C_L averaged 5.6 per cent and duplicate measurements invariably agreed within 10 per cent. Details of the lung mechanics measurements are described elsewhere (24).

[1] Godart-Mijnhart, Utrecht, Holland.

Volume-pressure relationship over an extended range. On three occasions in three animals the volume-pressure relationship of the lung over an extended range was studied by a method similar to that described by Cook and co-workers (29). The measurements were performed after the injection of 0.20 ml per kg barium sulfate while the animals were paralyzed with succinylcholine. Increments of 150 ml were introduced in 1 second with 5 seconds between each step. The transpulmonary pressure was measured with a Sanborn differential pressure transducer 267 B. Inflation was continued until the transpulmonary pressure was approximately 30 mm Hg greater than in the resting position. The gas was then removed in a stepwise fashion at the same time intervals as for the inflation. An approximate correction for change in gas volume from ATP to BTPS and for gas exchange was made by taking the volume increments as 160 ml BTPS for both inflation and deflation. *Statistical methods* were used as recommended by Snedecor (30). *Embolic material* consisted of a 33 vol/vol emulsion of barium sulfate injected into the pulmonary artery.

Groups. The animals were divided into two main groups. Group I (Table II), consisting of 19 animals, was given 0.20 ml per kg barium sulfate. Group II

TABLE II

Cardiorespiratory effects of the administration of 0.20 ml per kg barium sulfate emulsion in sheep *

Group I	†	Intact	Vagot.	Atrop.	Bretyl.	Lysergic acid
No. of animals		6	3	4	2	4
Cardiac output	0	3.12	3.50	3.29	2.75	3.19
($L/min/m^2$ *BSA*)	5	3.12	4.13	3.42	2.91	3.06
	30	2.87				
Femoral art. mean	0	123	107	118	115	125
pressure (*mm Hg*)	5	135	111	123	120	128
	30	109				
Syst. art. resistance	0	3,289	2,439	2,999	3,449	3,179
($dynes\text{-}sec\text{-}cm^{-5}/m^2$ *BSA*)	5	4,014	2,408	2,950	3,477	3,392
	30	3,169				
Pul. art. mean	0	13	15	17	14	16
pressure (*mm Hg*)	5	*32*	30	32	25	29
	30	24				
Pulm. art. resistance	0	344	357	435	432	396
($dynes\text{-}sec\text{-}cm^{-5}/m^2$ *BSA*)	5	*896*	693	776	717	753
	30	670				
Art. oxygen saturation	0	91.6	90.9	77.1	81.1	78.0
(%)	5	*81.7*	79.4	68.8	73.5	76.4
Venous admixture	0	13	16	37	30	37
(% *cardiac output*)	5	*33*	39	47	39	38
	30	45				
Ventilation	0	6.4	10.5	16.5	8.2	9.2
($L/min/m^2$ *BSA*)	5	*11.7*	12.1	19.6	12.2	15.3
	30	8.9				
Breathing rate (*min*)	0	30	31	60	41	43
	5	*48*	33	83	56	57
	30	50				
Tidal volume	0	219	338	271	198	226
(ml/m^2 *BSA*)	5	253	370	248	216	272
Lung compliance	0	3.49	2.58	3.33	2.52	2.90
($ml/cm\ H_2O/kg$)	5	*0.75*[21]	0.79[31]	0.83[25]	0.34[13]	0.99[34]
	30	1.06[33]				
	I	1.51[43]	1.10[43]	1.19[36]		1.51[52]
Mean airway resistance	0	2.21	1.82	1.20		
($cm\ H_2O/L/sec$)	5	*5.81*	4.60	2.94		

* Italicized figures = significant difference ($p < 0.05$). Numbers in brackets attached to compliance measurements = values in per cent of control.

† 0 = control period; 5 and 30 = 5 and 30 minutes after the injection of embolic material; *I* = after inflation of the lung.

(Table III), 28 animals, was given 0.02 ml per kg of the same substance, 0.1 of the previous dose.

After a control period (0) six animals of group I and 5 of group II were injected with barium sulfate. Pressures were continuously measured; other parameters were measured 5 minutes later. In some animals of group I, measurements were again repeated at 30 minutes. After the last measurement the lungs were inflated to a pressure of 30 mm Hg and C_L was again determined (*I*, Tables II and III). The remaining animals were subjected to various procedures prior to the control period.

Bilateral cervical vagotomy was carried out in 7 sheep; 0.2 to 0.4 mg per kg atropine sulfate was administered intravenously to 4 vagotomized and 4 intact animals. Ten mg per kg bretylium tosylate (Darenthin) was administered intravenously to 2 animals 30 minutes prior to the control period. N,N,N′,N,-3-pentamethyl-N,N′,-diethyl-3-aza-pentane-1-5-diammonium-dibromide (Pendiomid) was injected intravenously into 1 animal in a dose of 2 mg per kg; 100 to 150 mg promethazine hydrochloride (Phenergan, May and Baker) was intravenously administered to 5 animals 30 minutes prior to the control period; 0.5 mg per kg lysergic acid butanolamide (Deseril) was injected intravenously into 4 sheep 30 minutes prior to the control period.

In 3 animals control measurements of lung mechanics were followed by continuous breathing of 100 per cent oxygen. After an equilibration period of 10 minutes, circulation measurements were taken, barium sulfate administered and at 5 minutes, circulation measurements were repeated while the animals were still breathing oxygen. Oxygen breathing was then discontinued and measurement of lung mechanics repeated.

A continuous infusion of adrenalin (0.4 μg per kg per minute) was administered to 4 animals; a continuous infusion of isoproterenol hydrochloride (Isuprel; 0.03 μg per kg per minute) to 6 others. Control measurements and embolization were performed during the infusion.[2]

[2] Darenthin was supplied by Burroughs Wellcome & Co. (Aust.) Ltd.; Pendiomid by Ciba Co., Pty. Ltd.;

TABLE III

Cardiorespiratory effects of the administration of 0.02 ml per kg 33% barium sulfate emulsion in sheep *

Group II		Intact	Vagot. + atrop.	Pendiomid†	Oxygen 100%	Promethazine	Adren.	Isoproterenol
No. of animals		5	4	1	3	5	4	6
Cardiac output	0	3.93	2.91	5.31	3.27	3.64	5.16	5.00
(*L/min/m²*)	5	4.09	3.73	4.93	3.80	4.37	5.84	5.09
Femoral art. mean	0	120	107	115	131	102	133	76
pressure (*mm Hg*)	5	117	111	127	136	107	145	71
Syst. art. resist.	0	2,468	2,970	1,732	3,218	2,497	2,172	1,216
(*dynes-sec-cm⁻⁵/m²*)	5	2,489	2,433	2,062	2,912	2,057	2,001	1,148
Pulm. art. mean	0	15	16	16	15	19	17	9
pressure (*mm Hg*)	5	*20*	23	30	25	22	20	10
Pulm. art. resist.	0	302	440	241	381	446	271	153
(*dynes-sec-cm⁻⁵/m²*)	5	*384*	506	487	514	414	280	169
Art. oxygen sat.	0	90.4	84.2	86.9	105.9	81.3	88.8	93.8
(%)	5	86.8	81.0	69.7	105.5	72.0	88.0	94.0
Venous admixture	0	16	26	27	18	33	29	8
(*% cardiac output*)	5	22	30	50	19	45	32	8
Ventilation	0	8.7	10.8	16.9	8.0	14.5	10.6	11.0
(*L/min/m²*)	5	*12.3*	11.8	23.8	10.2	19.0	13.5	11.0
Breathing rate	0	35	26	58	31	48	33	35
(*min*)	5	*44*	26	71	41	53	39	34
Tidal volume	0	253	415	288	250	304	317	302
(*ml/m²*)	5	*284*	447	334	243	359	338	293
Lung compliance	0	3.13	3.24	4.10	4.41	3.01	4.14	4.02
(*ml/cm H_2O/kg*)	5	*1.46*[47]	1.78[55]	0.67[16]	1.96[44]	1.91[63]	2.94[71]	4.22[105]
	I	3.16[101]	2.66[82]	3.69[90]	3.24[73]		4.38[106]	
Mean airway res.	0	2.15						
(*cm H_2O/L/sec*)	5	3.06						

* See footnote to Table II.
† Ciba; see formula in Methods.

At the end of the experiments the lungs were in some cases removed and macroscopically assessed for edema.

RESULTS

Results are summarized in Tables II and III.

A. Intact animals

1. Effects on circulation. $\dot{Q}$ remained practically unchanged in both groups. The slight increase in $\overline{P}_{f.a.}$ and R_{syst} at 5 minutes in group I was significantly different ($0.02 < p < 0.05$) from the slight decrease in group II. The injection of the embolic material was followed by a rapid rise in $P_{p.a.}$. The peak value was reached within 30 to 60 seconds (Figure 1). At this stage the difference between the average peak values obtained after the injection of 0.20 ml per kg (= 52 mm Hg) and 0.02 ml per kg (= 44 mm Hg) was not statistically significant ($p = 0.30$). From then on $\overline{P}_{p.a.}$ gradually declined and at 5 minutes the difference between the two groups was statistically significant ($p < 0.01$).

2. Effect on arterial oxygen saturation and ventilation. Sa_{O_2} decreased significantly in group I only. There was a significant rise in $\dot{V}E$ in both groups; the values were higher in group I. The increase in f was significant in group I only. VT increased in both groups, significantly in group II only.

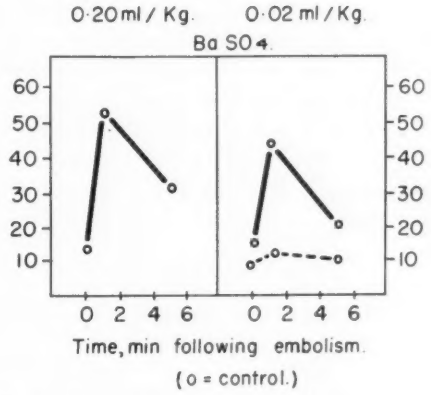

FIG. 1. AVERAGE VALUES OF THE MEAN PULMONARY ARTERIAL PRESSURE BEFORE (O), 1 MINUTE AND 5 MINUTES AFTER THE INJECTION OF 0.20 AND 0.02 ML/KG BARIUM SULFATE IN UNTREATED (SOLID LINES) AND ISOPROTERENOL-TREATED (DOTTED LINE) ANIMALS. Pressures in mm Hg.

3. Effect on mean frictional resistance. A statistically significant rise, which averaged 250 per cent, occurred in group I only. The administra-

Deseril by Sandoz (Aust.) Pty. Ltd.; and Isuprel by Winthrop Labs. Their courtesy is gratefully acknowledged.

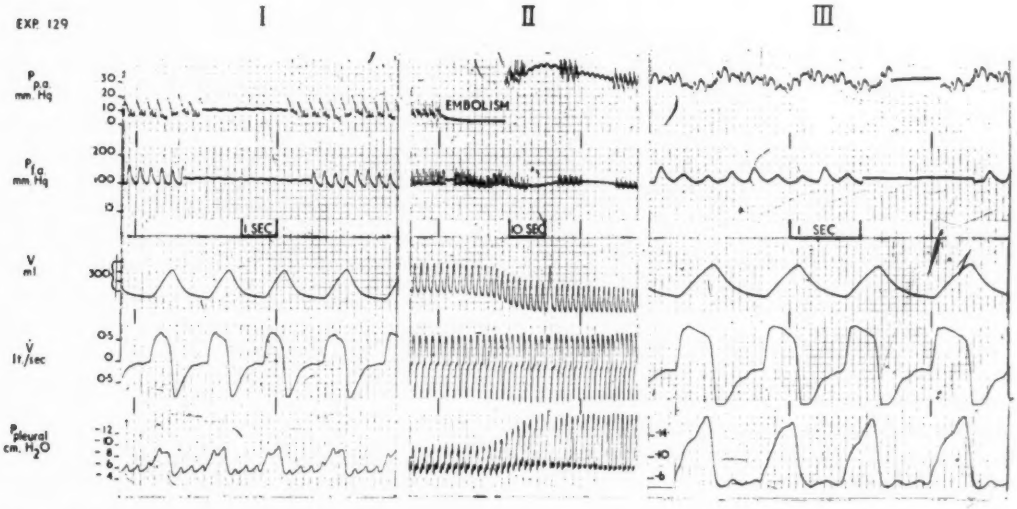

FIG. 2. INSTANTANEOUS EFFECTS OF EXPERIMENTAL PULMONARY EMBOLISM. I. Control measurement. II. Embolism. Note the instantaneous increase in the negativity of intrapleural pressure. III. Three minutes after embolization. $P_{p.a.}$ = pulmonary arterial pressure. $P_{f.a.}$ = femoral arterial pressure. V = integrated volume. $\dot{V}$ = air flow (pneumotachygraph).

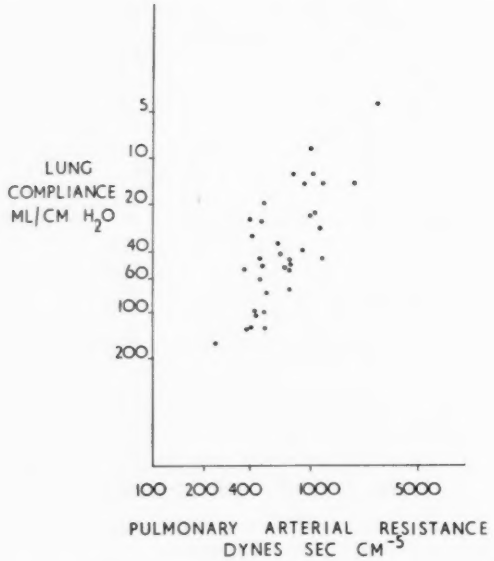

FIG. 3. RELATIONSHIP OF LUNG COMPLIANCE AND PULMONARY ARTERIAL RESISTANCE IN LUNG EMBOLISM. Black dots represent sheep subjected to barium sulfate embolization. Open circles indicate measurements taken in spontaneous pulmonary embolism due to blood clots.

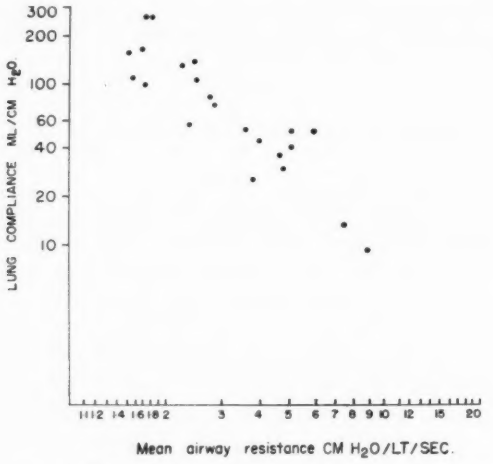

FIG. 4. RELATIONSHIP OF LUNG COMPLIANCE AND MEAN FRICTIONAL ("AIRWAY") RESISTANCE IN EXPERIMENTAL PULMONARY EMBOLISM.

tion of the smaller dose resulted in a lesser rise not statistically significant.

4. Effect on lung compliance. Embolism was followed by a gross fall in C_L, as illustrated by Figure 2 and Tables II and III. The fall was more severe after the large dose and increased only slightly throughout the first 30 minutes. Inflation to 30 mm Hg produced a variable improvement in group I but restored C_L in group II.

A definite correlation was obtained between R_{pulm} and C_L (Figure 3). A decrease in C_L was associated with an increase in R_{pulm}.

A further correlation was established between C_L and mean frictional resistance (Figure 4). A sizable rise in the latter occurred only with a gross fall in C_L.

Effective capillary blood flow ($Q_E = 100 - Q_S$) was plotted against values of C_L between the range of 10 to 60 ml per cm H_2O (Figure 5). The regression coefficient (+ 0.47) would have occurred by chance less frequently than 1 in 100 times ($p < 0.01$).

5. Effect on lung volume. During the first 20 seconds after the injection of the embolic material, air was expelled from the lungs, as illustrated by the volume trace on Figure 2. This was also confirmed by spirometer records not shown here. Parallel to this change there was an increase in end-expiratory transpulmonary pressure—i.e., the P_{pl} became more negative, as shown by the rise

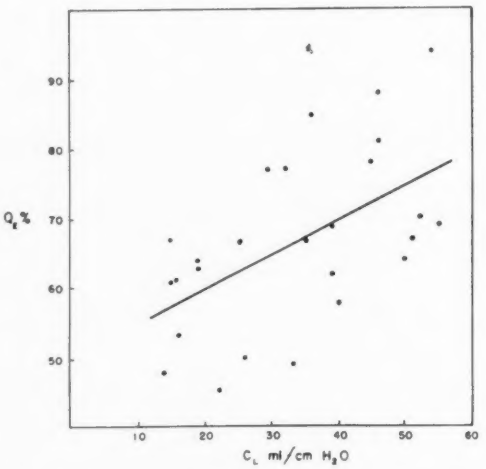

FIG. 5. RELATIONSHIP OF EFFECTIVE PULMONARY CAPILLARY BLOOD FLOW (Q_E) IN PER CENT OF TOTAL PULMONARY ARTERIAL BLOOD FLOW AND LUNG COMPLIANCE (C_L) IN EXPERIMENTAL PULMONARY EMBOLISM 5 MINUTES AFTER INJECTION OF THE EMBOLIC MATERIAL. Only animals with a compliance value of less than 60 ml/cm H_2O were included.

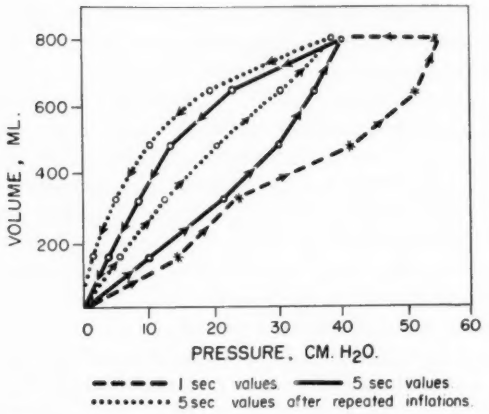

FIG. 6. VOLUME-PRESSURE CURVE OVER AN EXTENDED RANGE IN AN EMBOLIZED LUNG (0.20 ML/KG BARIUM SULFATE). For explanation see text.

in the expiratory level of the P_{pl} trace (bottom line) on Figure 2. The amount of air expelled was 150 to 200 ml. The end-expiratory transpulmonary pressure gradually returned toward the control level.

6. Volume-pressure relationship over an extended range. The result of a typical experiment is shown in Figure 6. The solid-line loop in the middle represents the volume-pressure curve measured at 5 seconds corresponding to the interval between successive volume steps. The curve for the pressure at 1 second lies to the right. There was a small fall in pressure between 1 and 5 seconds for each volume step in the tidal range. At higher levels of inflation the fall in pressure between 1 and 5 seconds increased to 10 to 15 cm H_2O. The total lung volume at 50 cm H_2O inflating pressure was still markedly reduced. After inflation the slope of the 5-second volume-pressure curve increased somewhat, but the total lung volume remained unchanged. Compliance, taking a pressure value between 1 and 5 seconds, equaled 13.4 ml per cm H_2O. The compliance measured during spontaneous breathing was 15.2 ml per cm H_2O.

7. Postmortem findings. Extensive lung edema was found in only one animal in group I.

B. Animals subjected to neuroplegic procedures

In vagotomized animals, f was lower than in the intact ones and failed to rise after embolism. V_T was higher and the rise in $\dot{V}_E$ was less than in the controls. Mean airway resistance was lower in the atropine-treated group. The postembolic rise, expressed as per cent of control value, was not different from that of the untreated group. With these exceptions the embolic response in these groups was essentially similar to that seen in the intact animals.

C. Other procedures

Lysergic acid butanolamide. The only difference in the response was a lesser rise in $\dot{Q}_S$.

Oxygen breathing. The response was unchanged.

Promethazine. This group had the highest initial $P_{p.a.}$ and R_{pulm}. Embolism produced the same initial rise in $P_{p.a.}$ as in the controls; at 5 minutes, however, $\bar{P}_{p.a.}$ was only slightly elevated. Because of a simultaneous increase in $\dot{Q}$, no rise in R_{pulm} occurred. The rise in $\dot{Q}$ was not statistically significant ($0.05 < p < 0.10$). The difference between the change in $\dot{Q}$ in this group and in the corresponding control group was not statistically significant ($p = 0.30$).

Adrenalin. The adrenalin infusion resulted in a gross rise in $\dot{Q}$ and in some increase in $\bar{P}_{f.a.}$. Both increased further after embolism. The postembolic rise in $\bar{P}_{p.a.}$ was considerably less than in the corresponding control group and R_{pulm} remained unchanged. The fall in C_L was less than in the preceding groups.

Isoproterenol. Infusion with this substance caused a gross rise in $\dot{Q}$, a fall in systemic and pulmonary arterial pressure and resistance, and a rise in Sa_{O_2}. Embolism was followed by a marginal rise in $\bar{P}_{p.a.}$ and virtually no change in R_{pulm}. The initial peak rise in $\bar{P}_{p.a.}$ was also largely abolished (Figure 1). Changes in $\dot{Q}_S$, $\dot{V}_E$ and C_L were completely prevented.

DISCUSSION

1. Ventilation. These experiments have thrown some doubt on the view that hypoxemia contributes to the ventilatory response to embolism as suggested by previous workers (12); the response was not significantly affected by 100 per cent oxygen breathing. Contrary to the widely held belief, V_T increased in both intact groups and decreased only after the repeated injection of large doses of

embolic material. Vagotomy or the administration of atropine, or both, reduced but did not abolish the response, while bretylium, lysergic acid butanolamide, promethazine, or adrenalin was without effect. Hyperventilation was completely abolished by isoproterenol infusion.

2. Lung mechanics. The most surprising change was the gross fall in C_L, the extent of which appeared to be related to the amount of embolic material injected. C_L is the sum of compliances of all distensible units within the lungs. When part of the lung was removed C_L showed a proportional fall (31). A reduction of the number of distensible units due to surface tension forces created by the alveolar transudate in lung edema was shown to be responsible for the fall in C_L (*29*, *32*). A gradual collapse of the terminal airways was suggested to account for a part of the spontaneous fall in C_L in anesthetized dogs (33). This mechanism, the sudden exclusion of a significant portion of the distensible units, appears to be the only possible cause for a gross and rapid fall in C_L; changes in lung blood volume would account only for minor changes (34, 35).

Pulmonary edema has occurred under certain conditions (36) and after a variable latent period (*37*) in lung embolism. Postmortem inspection of the lung revealed edema in only one of our animals. Further evidence against the presence of edema is the effect of inflation. In the presence of fluid in the airways, large inflations tend to promote its distribution, resulting in no improvement or a further drop in C_L (24). Inflation of the lung had a variable effect in group I (intact) and restored C_L in group II (intact). A normal C_L, a few minutes after embolization, is incompatible with lung edema.

The increase in mean frictional resistance was remarkably well correlated with the fall in C_L. This may represent flow through a smaller number of patent airways which in effect reduces the mean diameter of the sum of airways through which flow occurs. However, it is likely that part of the increase was due to bronchoconstriction.

Otis and colleagues (38) have demonstrated that the distribution of ventilation is influenced by breathing frequency if the time constants (products of compliance and resistance) of the separate areas in the lung are different. The problem, therefore, is how much of the fall in C_L is due to the inclusion of a dynamic element, in the measurement after bronchoconstriction, that was not present before.

The dynamic element in vagotomized animals is largely abolished by the inspiratory pause; the fall in C_L was, however, unchanged. The increased elastic resistance could also be demonstrated under semistatic conditions, as shown by the volume-pressure curve. This inflation through an extended range also indicates that the increase is not restricted to the tidal range. The agreement between C_L values taken under semistatic conditions and during spontaneous breathing indicates that the fall in C_L after embolism has not been overestimated.

A gross fall in pressure in the interval between inflations occurred only at higher levels of inflation. This indicates three possible causes: *1*) surface hysteresis, *2*) tissue relaxation, *3*) opening of distensible units.

The behavior of the embolic lung differs from that of the edematous lung in which one observes an earlier increase in slope, and the volume at 30 cm H_2O pressure is not much different from the normal lung (*29*). This suggests that the increased resistance to inflation is not due to surface forces and is consistent with a hypothesis that the alteration in lung mechanics is due to airway closure.

The airway musculature in man and animals extends over both the proximal (supported) and distal (unsupported) parts of the bronchial tree, with muscular rings surrounding the orifices of the atria (39), also referred to as "alveolar sphincters" (40). Muscular contraction of the proximal part of the airways, if unaccompanied by hypersecretion or mucosal swelling, or both, can only cause a narrowing of the lumen; the presence of cartilagenous rings prevents complete occlusion. Muscular contraction in the distal unsupported segments would result in airway closure with exclusion of the more peripheral alveoli from expansion. This suggested action might be facilitated by the thickness of these muscular bands; they are relatively five times thicker than the muscle rings of the cartilagenous bronchi (39).

It is suggested that pulmonary embolism is followed by a contraction of the smooth muscles of the airways. In the proximal segments this contraction causes some narrowing in the lumen, as

indicated by the moderate rise in mean frictional resistance. In the distal segments the same contraction would cause widespread closure of the terminal airways with a fall in ventilated lung volume and C_L. This appears to be the "unknown mechanism" (41) that produces a shift of ventilation toward the unaffected parts of the lungs, as demonstrated by fluoroscopy (42) and bronchospirometry (43) and by means of a balloon-catheter (44, 45).

The fall in C_L after embolization was accompanied by a decrease in lung volume amounting to 150 to 200 ml. If muscular contraction squeezed out air one would expect the end-expiratory P_{pl} to become more negative. Since compliance of the chest wall in the sheep is on the average 150 ml per cm H_2O (24), the expected rise in end-expiratory transpulmonary pressure could not exceed 1 to 2 cm H_2O. This was the change actually observed in the first half-minute following the embolus. Its subsequent return to normal can be accounted for by a gradual further expansion of the ventilated areas of the lung. In other cases the return to normal was apparent rather than real owing to active expiration, the P_{pl} at the end of expiration at the moment of zero flow remaining more negative than before embolism.

Robin (8) and Julian (44), and their associates, observed an increased alveolar dead space based on a widened alveolar-arterial P_{CO_2} difference in pulmonary embolism. In view of the gross mechanical nonhomogeneity of the lung after embolism, alveolar sampling is invalid. This may account for the gross variation in their results.

Because of the tendency of the anesthetized animal to breathe at a fixed V_T—i.e., constant transpulmonary pressure swing—C_L is likely to remain reduced until a large inflation is performed. The apparent duration of the stimulus responsible for the fall in C_L can therefore only be assessed by the period of time that must elapse after embolization before C_L can be restored by inflation. This period varied with the amount of embolic material injected. With larger doses, 30 minutes was not sufficient; with small doses, C_L was successfully restored a few minutes after embolization.

The inability of vagotomy and atropine to prevent the embolism-induced rise in mean frictional resistance is at variance with the findings of Binet and Burstein (5) and Boyer and Curry (6) and confirms the observations reported by Singh (4). The parasympathetic nervous system is apparently not involved in this change.

The fall in C_L remained unaffected by neuroplegic procedures and by the administration of antihistaminic and antiserotonin drugs. It seems safe to conclude that the suggested constriction of the airway musculature is unrelated to neurogenic stimuli and to the release of histamine or serotonin. The fall following the small dose of embolic material was reduced by adrenalin and completely abolished by isoproterenol. This proved the functional nature of airway closure and appears to suggest that some yet unidentified substance, causing a constriction of the airway musculature, might be released as a response to embolism.

The severe fall in C_L as demonstrated in these experiments is bound to increase the strain of the respiratory muscles already burdened by hyperventilation. We suggest that the resulting increase in the work of breathing could well be responsible for the severe dyspnea seen in some cases of clinical pulmonary embolism. Since the administration of isoproterenol abolished both hyperventilation and the rise in elastic resistance, it is likely to be beneficial in patients.

3. Arterial hypoxemia. Closure of the terminal airways, if relatively more extensive than obstruction of the vessels, may result in the perfusion of nonventilated alveoli with consequent venous admixture. This explanation is consistent with the inability of 100 per cent oxygen to abolish venous admixture (8, 9), and appears to be supported by the partial correlation between Q_E and C_L (Figure 5).

4. Pulmonary hypertension. The sudden steep rise in $\bar{P}_{p.a.}$ after the injection of embolic material (Figure 1) does not appear to be dependent on the amount injected. The extraordinary rise in $\dot{Q}$ that would be necessary of itself to cause such a rise is unlikely to have occurred. It is more probable that the rise represents a time lag between the embolism-induced obstruction and the opening of reserve capillaries. In this case one would expect the rise to be reduced or largely absent after repeated doses of emboli, but this is not the case; an equal initial rise was regularly observed with repeated injections. It is therefore suggested

that this sudden steep rise represents pulmonary vasoconstriction.

The argument most frequently used against the occurrence of embolic pulmonary vasoconstriction is mainly based on the absence of pulmonary hypertension following lobar emboli (18). This implies that local embolism does not cause generalized vasoconstriction in the lungs. The situation is analogous in the systemic circuit where localized emboli also fail to cause a generalized vascoconstriction. Lobar emboli are, however, of little assistance in excluding the occurrence of a local response, i.e., constriction of vessels actually containing the embolic particles. This mechanism is consistent with the dose-response relationship and would also explain how a relatively small dose of particles, as in our group II, can cause pulmonary hypertension.

The lack of effect of neuroplegic procedures to prevent postembolic pulmonary hypertension has been confirmed in these experiments. Even 100 per cent oxygen, an otherwise potent pulmonary vasodilator, proved ineffective. The lack of protection by lysergic acid butanolamide suggests that serotonin release is probably not involved in this mechanism.

The administration of promethazine failed to affect the immediate pressure response but reduced the 5-minute values considerably. Changes in lung mechanics were not influenced. This finding, therefore, does not provide evidence for the release of histamine, which is known to produce bronchoconstriction but no pulmonary hypertension (46).

The effect of adrenalin and even more the effect of isoproterenol provide conclusive evidence that pulmonary hypertension after the small dose of barium sulfate is predominantly functional. Isoproterenol has been demonstrated to be a potent pulmonary vasodilator in animals (47). In patients suffering from various types of heart disease isoproterenol in considerably smaller doses reduced R_{pulm} by increasing $\dot{Q}$; $P_{p.a.}$ remained unchanged (48). It is not clear whether this difference is due to the lower dose or to the different mechanism of pulmonary hypertension in those patients.

From these observations isoproterenol emerges as a substance which, if administered in high doses and continuously, effectively prevents all major consequences caused by the intravenous administration of 0.02 ml per kg barium sulfate. No other substance is known to offer a similar protection. It remains to be decided how effectively this drug can block the effects of larger doses of embolic material and what its action is if administered after embolism.

SUMMARY

Experimental pulmonary embolism was produced in sheep, by means of graded amounts of a barium sulfate emulsion. Ventilation, lung mechanics and circulation were measured. The effect of various neuroplegic procedures, oxygen breathing, the administration of antihistaminic and antiserotonin drugs, and continuous adrenalin and isoproterenol infusions was assessed.

In addition to the already known consequences of pulmonary embolism (hyperventilation, arterial hypoxemia, pulmonary hypertension and bronchoconstriction), a gross fall in lung compliance was shown to occur, unrelated to lung edema.

All but one of the procedures used in these experiments were ineffective in altering the onset or severity of the above changes, but the effect of the smaller dose of embolic material was completely prevented by the administration of isoproterenol.

It is concluded that postembolic pulmonary hypertension and compliance-fall after a small dose of embolic matrial are predominantly functional and probably caused by the release of an unknown substance as a response to embolism. The physiological and clinical implications of these findings are discussed.

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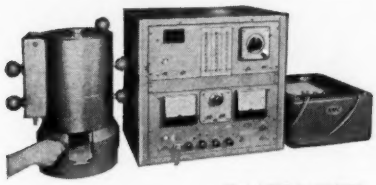

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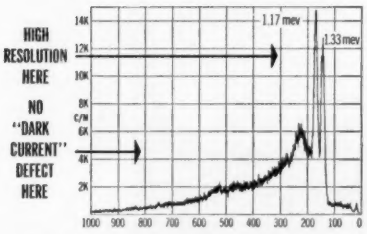

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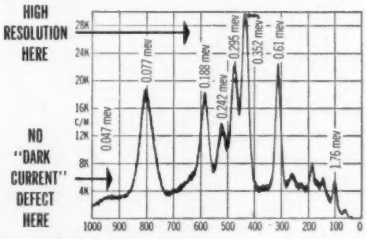

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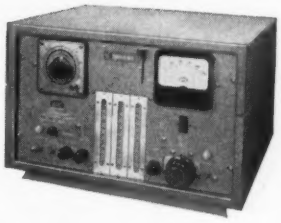

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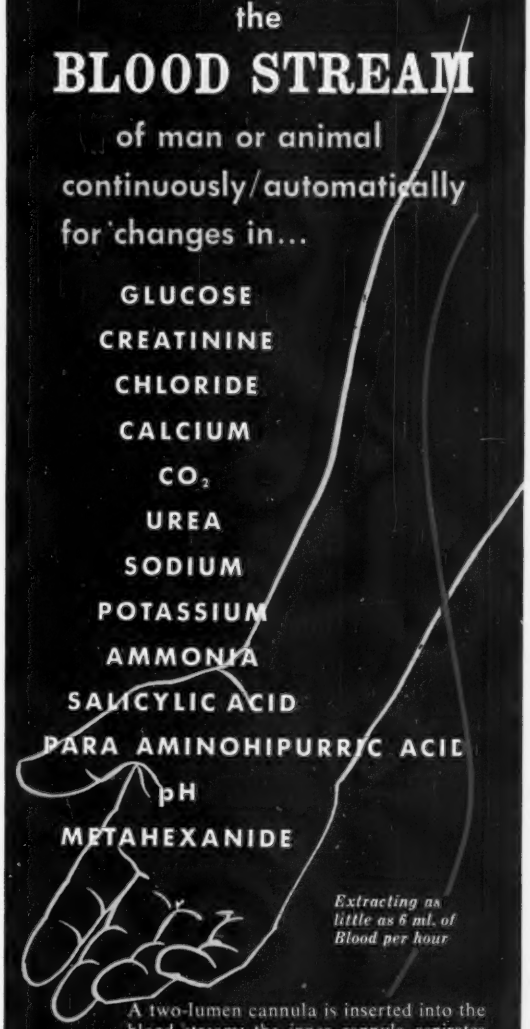

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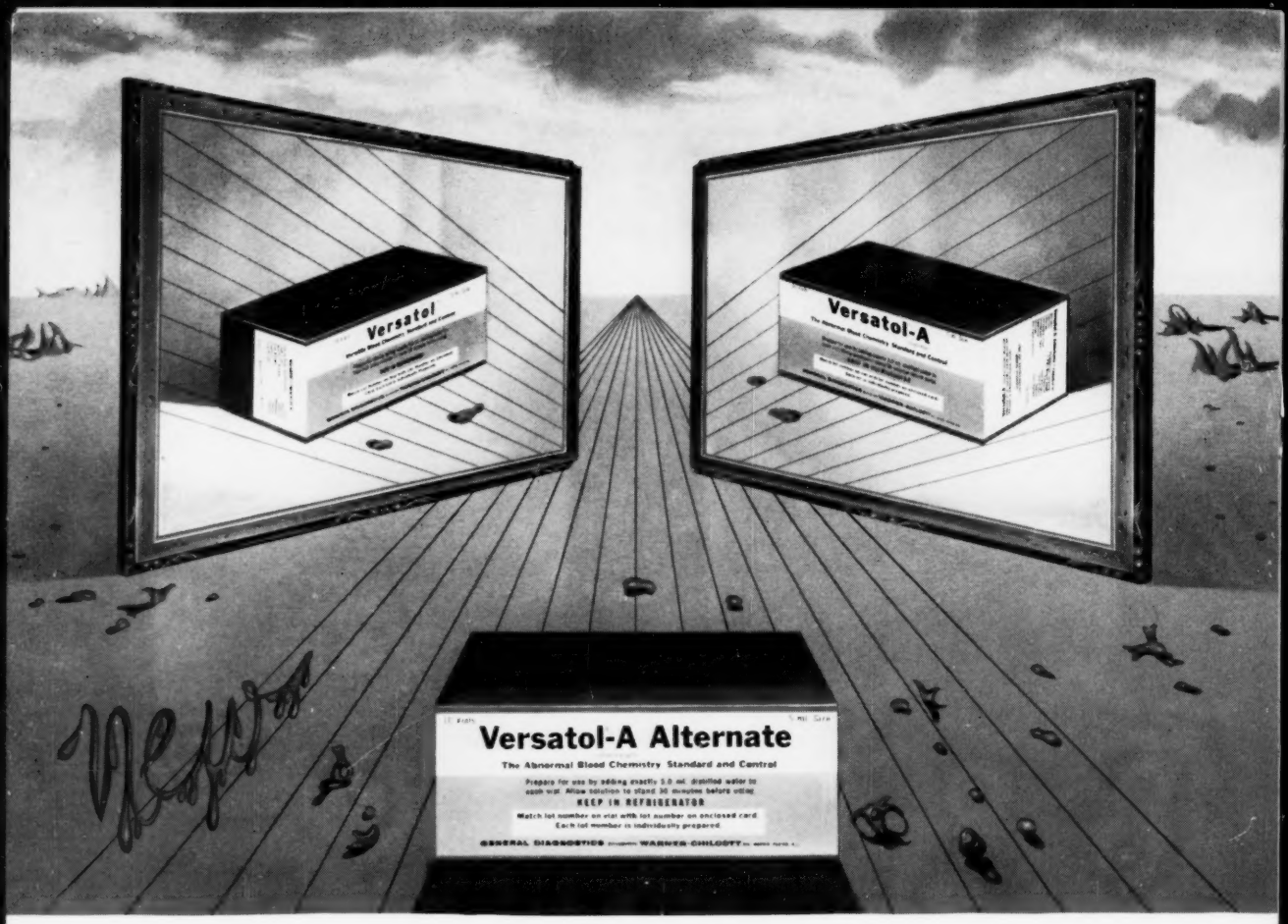

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